Studies on the bacterial populations of two-phase systems

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STUDIES ON THE BACTERIAL POPULATIONS OF TWO-PHASE SYSTEMS

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STUDIES ON THE BACTERIAL POPULATIONS OF TWO-PHASE SYSTEMS.

Abstract of Thesis submitted by T. R. Kamakaka.

A large number of papers have been published on the survival of organisms in aqueous systems, but comparatively little has been published on the survival of organisms in fixed oils. A critical review is given of the techniques employed by previous workers and the results obtained by them.

Fixed oils are shown to be highly bactericidal to dry Bacterium coli and mildly bactericidal to dry spores of Bacillus subtilis. A linear correlation has been found between the concentration of Bacterium coli in oil and the mortality rate. A coating of dried hydrophilic material is shown to protect Bacterium coli during freeze-drying, on subsequent storage, on treatment of the dried organisms with petroleum ether and against the bactericidal action of oils.

The rate of sedimentation, of dry organisms through light liquid paraffin, and their subsequent migration into an underlying aqueous phase suggests that suspensions of organisms in the oil consist of aggregates of organisms rather than free individual cells. A marked adhesion of the aggregates is observed to occur during sedimentation in the

oil. A technique is described for preparing more uniform suspensions of organisms in oils.

The majority of organisms, after sedimentation through oil, are adsorbed at the oil-water interface. This adsorption can, however, be reduced by lowering the interfacial tension between the two phases. A linear relationship is found to exist between the interfacial tension and the percentage of organisms passing from the oil to the aqueous phase.

The ability of some organisms to pass from an oil into an underlying aqueous phase suggests a new technique for counting viable organisms in oils. All the viable spores in an oil can be transferred to an aqueous phase by centrifuging the system at high speed, but the progressive rise in temperature of the system during centrifuging is observed to kill vegetative organisms. The proposed technique has the advantage over Bullock and Keepe's technique in that it avoids the use of bactericidal organic solvents.

Ringer's solution is found to be mildly bactericidal to Bacterium coli and oils are highly bactericidal. Nevertheless, when the two phases are in contact an initial

decrease in the number of viable organisms in the system is followed by an unexpected marked multiplication. The multiplication has been attributed to the ability of the organisms to multiply on soluble cellular eluates which are released into the system on the death of cells. The addition of low concentrations of eluate to Ringer's solution reduced the death-rate of Bacterium coli in that fluid.

CORRIGENDA

Page	Line	
12	After Table 1 line 1	for "vegative" read "vegetative"
33	12	for "dried organisms" read "Dried Organisms"
35	10	for "non-homogeniety" read "non-homogeneity"
108	1	for "0.0" read "0.06"
179	18	for "aqueous phase" read "the aqueous phase"
239	16	for "from oil to aqueous phase" read "from the oil to the aqueous phase"
245	Table 92	add the last line column 1 column 9 96 hours 2246.30
311		add between 3 and 4 "Bean, H.S., and Walters, V.(1955) J. Pharm. Lond. 7,661 Studies on bacterial populations in solutions of phenols. Part I. The viablity of Bacterium coli in aqueous solutions of benzylchlorophenol.

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SECTION I.

H I S T O R I C A L

INTRODUCTION

The survival of organisms in aqueous fluids has been extensively studied but comparatively little has been published on the fate of organisms in oily systems. Investigations that have been made on the survival of organisms in oils reveal inconsistent results, due no doubt, to the variations in technique employed by different workers.

Oils are liable to be contaminated during expression from oily seeds and fruits. Even so Lansberg (1920) found most commercial samples of oil to be sterile. He found samples of olive oil that had been contaminated to be self-sterilising, and recorded that the bactericidal power of different oils, and, even different samples of the same oil, varied considerably.

The pharmacist is interested principally in the sterility of oils intended for injections and in the bactericidal activity of such oils. They may be contaminated by dust particles bearing dried organisms, or by contact with a non-sterile syringe needle which may bear either dry organisms or moist organisms contained in a droplet of aqueous fluid. The physical environment of contaminating organisms must influence their survival in both aqueous and anhydrous systems.

(A) THE INTRODUCTION OF ORGANISMS IN OILY SYSTEMS.

One of the earliest attempts to introduce organisms into an oily system was made by Koch, cited by McMaster (1919). His method consisted of immersing silk threads into an aqueous suspension of spores of Bacillus anthracis, removing the threads from the suspension and drying them. The dried threads, with the spores adhering to them, were then immersed in the oils. The thread acted simply as a support for the spores and enabled the latter to be brought into direct contact with the oils. The spores remained attached to the threads throughout the immersion period and were never free as a suspension in the oils. Similar techniques were used by Laubenheimer, cited by McMaster (1919), Eisman, Jaconia and Meyer (1953), and by McMaster (1919). The latter subsequently changed his method of infecting the oils, because, when he assessed the viability of the organisms, he obtained irregular results. He attributed his irregular results to an unsatisfactory method of bringing the organisms into contact with the oils.

An attempt, to introduce organisms into fixed oils by adding a locpful of a 24-hour slope culture of

Bacterium Coli to a tube of sterile oil containing sterile sand, was made by Lichenstein (1939). The tube was stirred and shaken vigorously when the sand assisted the breakdown of clumps of organisms and promoted a more uniform dispersion in the oil. The organisms, as removed from the agar slope were moist and it is extremely probable that Lichenstein's system consisted of a dispersion in the oil of fine aqueous droplets containing the organisms. It is unlikely that the organisms ever came into direct contact with the oil. Similar techniques were used by Davies and Fishburn (1946) and Eisman, Jaconia and Meyer (1953).

Lichenstein's method was modified by Sykes and Royce (1950) who sludged agar slope cultures of Chromobacterium prodigiosum with a few millilitres of sterile oil and the suspension, thus produced, was subsequently diluted with further quantities of sterile oil. The suspension consisted of moisture coated organisms dispersed throughout the oil and, again, was not a true suspension of dry organisms in oil.

Even less satisfactory were the attempts of Leusden and Derlich (1940), Lembach (1947) and of Coulthard, Chantrill and Croshaw (1951) who attempted to inoculate various oils with aqueous suspensions of organisms. The suspensions of these workers were all dispersions of aqueous droplets containing organisms.

The above-mentioned workers appear to have taken it for granted that when moist organisms are introduced into an oil, the organisms migrate from the aqueous phase to the oily phase, where the oil will act directly upon the organisms. None of them appear to have attempted to prove this migration. This is surprising because solid particles wetted with water or other aqueous fluids, are not compatible with a hydrophobic phase. Thus the organisms in the systems described must have remained in the dispersed aqueous globules and were protected from any direct action of the oils. Any observed effect of the oils on the survival of the organisms in the systems described can only be attributed to the diffusion of water-soluble materials from the oil to the aqueous phase.

Lee and Chandler (1941), Fabian and Pivnick (1953), Pivnick, Engelhard and Thompson (1954) and Pivnick and Fabian (1954) added aqueous suspensions of organisms to cutting oils, which are petroleum oils emulsified to produce oil-in-water emulsions. The organisms introduced into such systems can be expected to remain in the aqueous phase, where the direct action of the oils was likely to be negligible.

Fairhall and Bates (1920) appear to be the first workers to attempt to infect oils with free, dry bacteria or mould spores. Unfortunately, they gave no details of their

method of drying the spores. Their oils were dried over calcium chloride or anhydrous sodium sulphate and it may be presumed that similar agents might have been used for drying the spores.

Coulthard, Chantrill and Croshaw (1951) infected oils with tubercule bacilli which were previously dried over a mixture of kaolin and talc at 37°C for 24 hours. It is doubtful, however, whether their bacteria were completely dry, as neither kaolin nor talc are good dehydrating agents.

Bullock, Keepe and Rawlins (1949) produced uniform spray-dried peptone powders containing either bacterial spores or vegative organisms. Later Bullock and Keepe (1951) used similar powders to infect sterile oils. The powders were lightly triturated with the oils to produce cloudy suspensions containing known concentrations of either Streptococcus faecalis or spores of Bacillus subtilis. Further attempts to produce almost clear suspensions of spores were made by introducing into oils, spray-dried stearin powder containing spores of Bacillus subtilis. The stearin dissolved in the oils leaving spores freely suspended. Thus Bullock and Keepe (1951) were the first workers to obtain a true suspension of dry organisms in oils.

Eisman, Jaconia and Meyer (1953) attempted to infect oils with vegative organisms by the method of Bullock and Keepe (1951) but failed to produce uniform susnensions.

(B) THE ASSESSMENT OF ORGANISMS IN OILY SYSTEMS.

I. In Vitro Quantitative Methods (Extinction Time Methods).

After known periods of immersion in the oils, Koch, cited by McMaster (1919), withdrew his silk threads with the dry spores adhering to them, transferred them to nutrient broth and incubated them at 37°C. Growth in the broth after incubation revealed the presence of one or more surviving spores on the thread. McMaster (1919) washed his threads with oil solvents after withdrawing them from the oils and before immersion in nutrient broth. It is not surprising that the results of McMaster were irregular, since Bullock and Keepe (1951) have shown that many oil solvents are highly bactericidal.

Lichenstein (1939) detected viable organisms in his systems by removing a loopful of the sand-oil suspension and transferring it to the surface of an agar slope. Leusden and Derlich (1940) transferred a loopful of infected oil to the surface of an agar plate.

Davies and Fishburn (1946) mixed five millilitres of contaminated oil with an equal volume of petroleum ether to reduce the viscosity of the former and filtered the mixed oils through a sterile Seitz filter pad. The filter

pad was thoroughly washed with petroleum ether and then incubated in nutrient broth. The authors recognised that the petroleum ether might have had a deleterous effect on the organisms. They attempted to determine the magnitude of this effect by adding a drop of a 24-hour broth culture of Staphylococcus aureus or Bacterium coli to five millilitres of petroleum ether, filtering the mixture through a sterile Seitz pad, and incubating the pad in nutrient broth. Growth after incubation of the pad indicated non-sterilization of the broth culture by the petroleum ether. They failed to recognise, however, that their organisms, being in a loopful of broth, probably never came into direct contact with the petroleum ether. Their test simply determined that no bactericidal agent in the fixed oils or petroleum ether diffused into the broth and exerted a bactericidal action.

Sykes and Royce (1950) employed a semi-quantitative method which involved the preparation of ten-fold serial dilution in sterile oil, of an oil infected by moist organisms. One millilitre of each dilution was added to 50 millilitres of broth and incubated at 37°C. After five days incubation subcultures were made into fresh broth to determine whether any turbidity present was due to bacterial growth or to oil globules in suspension.

The British Pharmacopoeia (1957) adopts a similar technique for "tests for sterility" of oils and oily solutions. It recommends that "when oily solutions or suspensions are being tested, they are distributed as uniformly as practicable throughout the media, and the medium is shaken at intervals during cultivation". The pharmacopoeial technique implies that the organisms will migrate from the oily to the aqueous phase, but the present author has been unable to trace any reference in the literature to the movement of free organisms between such phases. Bullock and Booth (1953), however, have demonstrated that spray dried peptone powders containing spores of Bacillus subtilis move from the oily to an aqueous phase when the two phases are simultaneously present.

II. In Vivo Qualitative Methods.

Samples of oil infected with Streptococcus haemolyticus were injected intra-peritoneally into mice by Coulthard, Chantrill and Croshaw (1951) and the number of deaths recorded after four days, the cause of death being confirmed by spleen culture. The same authors detected living tubercule bacilli in oils by injecting the infected oils into the peritoneum of guinea-pigs. The presence of living organisms was determined by (a) macroscopic examination of the spleen, liver, lungs and glands of the killed animals, (b) culture

of the spleen on Löwenstein medium and (c) the tuberculin test on living guinea pigs.

III. Quantitative Methods.

The silk thread method of Koch was modified by Eisman, Jaconia and Meyer (1953), who attempted to produce a quantitative method of assessing the effect of different oils on the viability of bacteria. Their infected silk threads were withdrawn from the oils, transferred to and thoroughly shaken with a dilute aqueous solution of Tween 80 (polyoxyethylene sorbitan monooleate)). Samples of the Tween 80 solution containing spores in suspension were plated out and incubated. The method was liable to considerable error because of the variable number of spores on different threads and also to variations in the number subsequently washed off the threads.

Bullock and Keepe (1951) mixed samples of oils infected with spray-dried peptone powder containing bacteria, with a suitable fat solvent (light petroleum ether for spores of Bacillus subtilis and anaesthetic ether for Streptococcus faecalis) and centrifuged the mixture to deposit the organisms. The supernatant fluid was decanted off and the deposit of the organisms twice washed with the same fat solvent, the last traces of which were removed after the second washing by

evaporation under reduced pressure. The residue was suspended in an aqueous diluent and a viable count performed on the suspension. The method was a marked advance over previous techniques. However, its application involves precise information on the effect of the fat solvent on the bacteria in question. The mortality of the organisms in the solvent depends on the nature of the bacteria, the time of contact between bacteria and solvent, the type of solvent used and probably the concentration of the organisms in the solvent. The method would, therefore, be unsatisfactory when applied to suspensions of unknown organisms in the oils, such as would be the case when enumerating chance contaminants of an oil. . .

(C) THE SURVIVAL OF ORGANISMS IN OILY SYSTEMS.

The consequence of different methods of introduction and assessment of organisms in oily systems is seen in the contradictory reports of various workers about the survival of organisms in such systems.

It was observed by Ritsert, cited by Lansberg (1920), that fats were bactericidal. This could not be confirmed by Achard and Foix, also cited by the same author. Degraeff (1921) reported that olive oil was able to dissolve tubercule bacilli, and that it did so ten times faster than castor oil. Lembach (1947) refuted the dissolution of organisms by oils, but found that the oils were bactericidal to bacterial suspensions added to them. Löhr, cited by Lichenstein (1939), reported cod-liver oil to be bactericidal, but found that organisms were able to survive in liquid paraffin for 14 days. He attributed the action of cod-liver oil to the high surface-tension which, he suggested, prevented organisms from multiplying. Had surface tension effects alone been responsible for this action, liquid paraffin should also have been bactericidal.

Leusden and Derlich (1940) observed that the survival time of organisms in cod-liver oil varied from

sample to sample and depended also on the species of the organisms. (Table 1).

TABLE 1.

THE SURVIVAL TIME OF DIFFERENT ORGANISMS IN COD-LIVER OIL.

Organism	Survival time
(1) Staphylococci, haemolytic streptococci, *Bacterium coli*, *Bacterium typhosus*	½ to 4 hours.
(2) *Corynebacterium diphtheriae*	10 minutes to 2 hours.
(3) *Bacillus subtilis* spores	2 hours.
(4) *Bacillus mycoides* spores	1 - 5 minutes.
(5) *Bacillus anthracis* spores	4 days.

Vegative forms of *Bacillus subtilis*, *Bacillus mycoides* and *Bacillus anthracis* were able to survive for only a few minutes. The authors made the important observation that emulsions of cod-liver oil were not bactericidal, whereas the oil itself possessed appreciable bactericidal action. It would appear that the high activity displayed by the pure cod-liver oil may be attributed to the low water-content of Leusden and Derlich's system. Any water-soluble bactericidal material

in the oil would be partitioned between the oil and the small water droplets containing the organisms, and would create a relatively high concentration in the water. Cod-liver oil emulsions contain a comparatively large proportion of water and a correspondingly small proportion of cod-liver oil. Thus the concentration of any water soluble bactericidal agent in the continuous phase of the emulsion will be much lower than in the small water droplets dispersed throughout the pure cod-liver oil. This lower concentration of water-soluble agent in the aqueous phase would presumably account for the much lower activity of the emulsions.

A clear denial of the bactericidal activity of most fixed oils, including cod-liver oil, was made by Goertzen (1935). His results could be explained on the grounds that his systems contained nutritive material. Lichenstein (1939) demonstrated that fixed oils of vegetable or animal origin were unquestionably more bactericidal than mineral oils.

Sykes and Royce (1950) found that Chromobacterium prodigiosum died off very rapidly in liquid paraffin, arachis oil and ethyl oleate. Coulthard, Chantrill and Croshaw (1951) observed that moist Streptococcus haemolyticus were less resistant to arachis oil or liquid paraffin at refrigerator temperature than at room temperature. The converse was true

with regard to moist tubercule bacilli. The latter authors further reported that dry tubercule bacilli were less resistant than blood covered moist organisms. The authors' results are, however, open to criticism in that the bacteria in their systems were heavily coated with nutritive material.

Eisman, Jacónia and Meyer (1953) recorded the multiplication of organisms in sesame oil. This multiplication must have occurred in the broth droplets distributed throughout the oil and not in the oil itself.

Bacteria were found to multiply by Lee and Chandler (1941) in freshly made soluble oil emulsions. Soluble oils are usually petroleum oils mixed with emulsifying agents, such as soaps of petroleum sulphonates, rosin, tall oil or fatty oils. These soluble oils are usually mixed with varying quantities of water to form stable and milky emulsions. The authors also made emulsions using fixed oils of vegetable and animal origin. They concluded that organisms will grow in emulsions made with any of the three types of oils and they do not require for growth any nutritive material other than that provided by the emulsions themselves. They also obtained growth in Locke's basal solution containing 1 per cent naphthenic acids and 0.1 per cent potassium nitrate. They concluded that naphthenic acids, which are present in mineral oil, supply the nutritive material in the soluble oil emulsions. Similarly Duffet, Gold and Weirich

(1945) reported the presence of organisms in numbers of up to 1 million per millilitre in soluble oil emulsions obtained from industrial sources.

Three further papers on the same subject have appeared since the present work was commenced. Fabian and Pivnick (1953) obtained the growth of up to 10^7 to 10^8 organisms per millilitre in 4 per cent soluble oil emulsions made with tap water. They obtained growth in soluble oil emulsions prepared from 13 different soluble oils. Growth also occurred in these emulsions after inoculation with 13 pure cultures obtained from three industrial soluble oil emulsions, as well as, with stock cultures of Aerobacter aerogenes, Pseudomonas aeruginosa and Bacterium coli. Typical growth curves, as obtained by the authors, are shown in Fig.I.

The authors observed that as the oil concentration in the emulsion was increased, the mean generation time in the logarithmic phase of growth decreased (Table 2 and Fig.II). They inferred that some component of the oil, present in relatively small concentration, supported growth.

The same authors also found that the addition of 0.5 per cent peptone to the soluble oil emulsions gave a hundred-fold increase in the number of organisms present after four days.

Very small numbers of coliform bacteria were found in soluble oil emulsions from industrial sources, although faecal bacteria were observed to grow well in them (Pivnick and Fabian

TABLE 2.

THE EFFECT OF OIL CONCENTRATION ON THE MEAN GENERATION TIME OF BACTERIA IN SOLUBLE OIL EMULSIONS. (FROM FABIAN AND PIVNICK - 1953).

Oil concentration.	Mean generation time.
10.00 %	173 minutes.
1.00 %	193 minutes.
0.10 %	247 minutes.
0.01 %	280 minutes.
0.00 %	367 minutes.

(1954). Pivnick, Engelhard and Thompson (1954) observed that some respiratory and enteric pathogens grew in soluble oil emulsions.

Fabian and Pivnick (1953) state that bacteria are not known to grow in straight oils, as water is necessary for the synthesis of cellular materials. In the above-mentioned results where growth has occurred as in all soluble oil emulsions so far examined, the prime factor required for the growth of bacteria, i.e., water, was present.

It has been shown above that a number of workers have

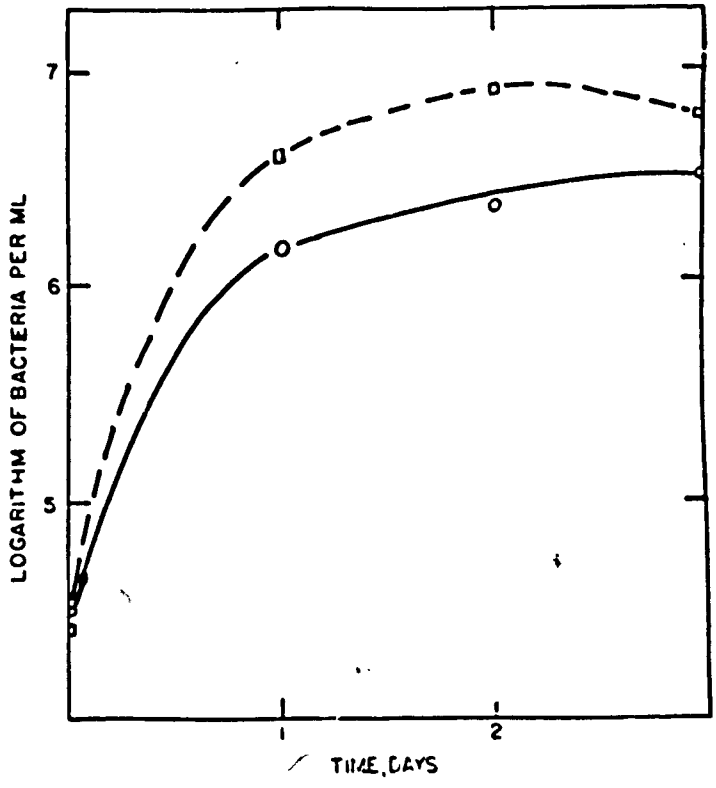


Fig.I. Growth Curves in Soluble-oil Emulsions of Two Isolates Obtained from Industrially-used Emulsions. These curves are representative of 13 Isolates studied. (Fabian and Pivnick - 1953).

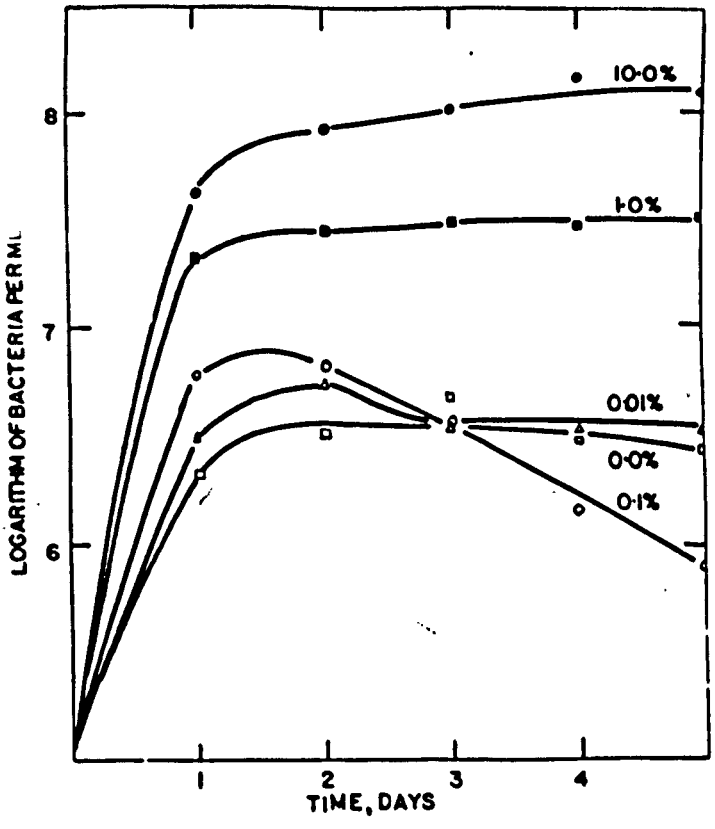


Fig.II. Effect of Oil Concentration on the Growth of Bacteria in Soluble-oil Emulsions. (Fabian and Pivnick - 1953).

examined the fate of organisms added to oily systems. It must, however, be emphasised that except for those workers who suspended organisms dried on silk threads in the oils, all the systems examined consisted of dispersions in the oils of small aqueous droplets containing organisms.

The first attempt to determine quantitatively the survival of freely suspended dry organisms in oils was made by Bullock and Keepe (1951). They found that while spores of Bacillus subtilis were not killed within six months in fixed oils, vegetative organisms, such as Streptococcus faecalis tended to die much more rapidly. The authors, however, thought that because the vegetative organisms also died rapidly in dry state, this observed mortality was not due to the action of oils.

(D) THE SURVIVAL OF ORGANISMS IN AQUEOUS SYSTEMS.

In contrast to the contradictory reports on the survival of organisms in oily systems, the reports dealing with their survival in aqueous systems are in much closer agreement. Many papers have been published on the survival of coliform bacteria in water from natural sources. The conditions in natural waters, however, vary considerably and hence the survival of the organisms varies enormously from water to water. Most of the literature mentioned below, on bacterial survival is qualitative in conclusion, the authors simply reporting the presence or absence of live organisms after specified periods of storage.

It has been suggested by many workers that vegetative organisms die off rapidly in water. Because of their high resistance to external conditions the spores of bacteria have been found to survive for a considerably longer time. Thus, Koch, cited by Topley and Wilson (1948), found that the spores of Bacillus anthracis remained viable for more than 90 days.

Among the factors which have been found to influence the survival of organisms in aqueous suspensions the following are of great importance :-

I The Source of the Water ..

Bacterium coli was reported to survive in distilled

water for several hours in undiminished numbers by Winslow and Brooke (1927), while the same authors reported that Bacillus cereus, Bacillus megatherium and Bacterium prodigiosum died off almost immediately when similarly treated. Wheeler (1906) contended that there was no difference between the survival rate of Bacterium typhosus in distilled water and in tap water, but polluted well water favoured survival, undoubtedly because of its content of organic matter. Rector and Daube (1917) observed a marked initial increase of Bacterium coli in water, followed by a gradual decrease. Suzuki (1941) stated that under most conditions, distilled water had a destructive action on Bacterium coli.

The deleterious effect of metallic impurities in the water, obtained from metallic stills has been shown by different workers since Ficker (1898) reported that water containing copper sulphate in a dilution of 1 in 50 million was sufficient to destroy Vibrio cholerae in 1 hour. Since then Wilson (1922), Hoder (1932), Davis (1940) and others have reported the bactericidal action of copper in distilled water on various organisms. Similarly, the harmful effect of alkalies liberated from glass containers has been reported by Ficker (1898) and Cohen (1922). Also traces of potassium dichromate in water have been found to be destructive to Staphylococcus aureus (Davis-1940).

II Hydrogen-ion Concentration of the Water

Winslow and Falk (1923) concluded from their results on Bacterium coli that the organism persisted in distilled water at pH 6.0 for 24 hours, with only slightly diminished numbers, but a sharp reduction in the number of organisms was recorded in waters which were more acidic than pH 5.0 or more alkaline than pH 7.0 (Table 3).

TABLE 3.

VIABILITY OF BACTERIUM COLI IN DISTILLED WATER AFTER 9 HOURS AT 37°C. (FROM WINSLOW AND FALK - 1923)

pH of distilled water	4.0	5.0	6.0	7.0	7.5	8.0
Percentage of organisms surviving	1	82	106	54	35	12

Cohen (1922) stated that the mortality of bacteria on storage at constant temperature in unbuffered solutions, e.g. distilled water, was variable and coincidental with apparently insignificant pH variations. At 20°C, Bacterium typhosum possessed the highest viability, when suspended in distilled water kept within the narrow pH range of 5.0 to 6.4. A slight increase in acidity to below pH 5.0 resulted in conditions of maximal mortality. For Bacterium coli, the pH range was wider and about neutrality. The author showed that the addition of buffer salts stabilised the variations in pH of the distilled water and consequently the variations in the viability of the organisms. Similarly, Zobell and Zobell (1932) found that hydrogen-ion concentrations above pH 8.0 and pH 6.6 rapidly shortened the period of viability of the organisms of the Brucella group. The optimal pH for these organisms was found to be between pH 7.0 and 7.4. Butterfield (1933) contended that for water-borne bacteria critical adjustment of the pH was not required, provided it was not above pH 8.2 and undiminished survival of the organisms was desired for only a very short period (30 to 60 minutes). He found that distilled water with pH about 9.0 was decidedly bactericidal.

III The Effect of Salts

Ficker (1898) was the first worker to show the

bactericidal effect of physiological salt solution. Sherman and Holm (1921) and Winslow and Falk (1923) found that the toxic effects of sodium chloride could be neutralised by calcium chloride alone. According to Winslow and Falk, the optimal ratio of sodium chloride: calcium chloride was 4:1 with a total concentration of 0.725 M. The protective effect was observed to be most beneficial in solutions having alkaline reactions. Boissenvain and Webb (1928) found the optimal concentration for viability of Bacterium coli to be $^{M}/_{4}$ of non-electrolytes and $^{M}/_{8}$ of binary electrolytes. The authors also concluded that cations were without effect on the viability of organisms except for a valency effect and that the HPO_4^{--} ion is the only mineral constituent necessary. In their solution containing a mixture of primary and secondary sodium phosphates at pH 7.0, the number of colon bacilli remained constant for a long period.

According to Ballantyne (1930) many organisms remained viable for 5 to 32 months in 0.85 per cent sodium chloride solution at various temperatures. He found that distilled water was more favourable for survival of Bacterium typhosus than 0.85 per cent sodium chloride solution. Zobell and Zobell (1932) observed that, contrary to expectations, the Brucella group of organisms lived longer in 0.25 per cent than in 0.85 per cent sodium chloride solution. The authors also showed that the addition of dilute mixtures of phosphates

and carbonates at pH 7.0 gave slightly better survival times. Further, the proportion of sodium or potassium ions and omission of either of them was observed to have no harmful effect, but an excess of calcium ions (0.1 per cent $CaCl_2,6H_2O$) was decidedly toxic. They also found that traces of calcium and magnesium had a stabilising influence on the viability of organisms, while nitrites and nitrates of sodium and potassium were toxic. Some of these observations were later supported by Butterfield (1933), who demonstrated that waters containing phosphates gave the highest and most consistent counts with Bacterium coli. On the other hand, Winslow and Brooke (1927) found that sugars and salts had no beneficial effect on the viability of Bacillus cereus, Bacillus megatherium, and Bacillus prodigiosum. Panisset, Verge and Carneiro (1925) observed the deleterious effect of 0.8 per cent sodium chloride solution on staphylococci, Bacterium typhosus, Bacterium coli, Bacillus anthracis and Friedlander's bacilli. Cook (1954) recorded that Pseudomonas pyocyaneus, Bacterium coli and Shigella dysenteriae remained relatively stable over a period of 50 hours in distilled water and in quarter-strength Ringer's solution. In fact, a small amount of growth was shown in the latter. Cook and Steele (1955) have recently reported that Bacterium coli remained unchanged in distilled water for 42 days. Hugo (1955) observed a substantial drop in surviving numbers of Bacterium coli in quarter-strength

Ringer's solution within a period of 12 days. Similarly, Fabian and Pivnick (1953) recorded that the colon group of organisms remain practically undiminished in numbers in phosphate buffer solution at pH 7.0.

IV The Effect of Organic Matter .

As early as 1898, Ficker reported an increase in the number of bacteria in a sample of stored water. This increase in the count followed an initial decrease, and was attributed by the author to the ability of surviving organisms to multiply at the expense of dead organisms. Boissevain and Webb (1928) concluded that the colon bacillus was apparently able to obtain its requirements of nitrogen from the air or from dead bacteria. Charred cotton fibres, which had fallen into the solution from the cotton plugs, were sufficient to supply the organisms' requirements of carbohydrate. Similarly, Davis (1940) observed the multiplication of Bacillus subtilis on heat-killed spores or eluate from them.

Wheeler (1906) obtained an appreciable multiplication of Bacterium typhosus in sterile well water containing organic pollution. A similar multiplication of Bacterium coli in drinking water stored at 37°C was observed by Fricker (1944).

Zobell and Zobell (1932) observed that the addition of organic materials such as serum, peptone, or meat extract improved a suspending fluid and initiated the multiplication of bacteria even at room temperature. Penfold and Norris (1912), Hucker and Carpenter (1927) and Friedlein (1928) reported that the minimal concentration of organic nutrients required for the multiplication of heterotrophic organisms ranges from 0.001 to 0.01 per cent or 10 to 100 milligrams per litre. Bigger (1937) and Bigger and Nelson (1941) supported this statement. The latter authors further suggested that the growth promoting substances found in water were carbon-dioxide and ammonia together with some insoluble inorganic substance which helped their adsorption. Butterfield (1928) found that after an extended lag period, Aerobacter aerogenes multiplied slowly in a solution containing only 0.5 milligram per litre each of glucose and peptone. A similar observation of the multiplication of Bacterium coli was made by Heukelekein and Heller (1940). Burke and Taschner (1936) reported the growth of Bacterium coli in the presence of traces of organic matter (0.05 p.p.m.), while Allen, Pasley and Pierce (1952) reported growth with 0.28 p.p.m. of organic matter. Marine heterotrophic bacteria were observed by Zobell and Grant (1943) to multiply in mineral solutions containing 0.1 milligram per litre of peptone or glucose. The authors were able to obtain a similar multiplication of Bacterium coli, Staphylococcus citreus, Bacillus megatherium, Lactobacillus

lactis and Proteus vulgaris in glucose solution containing 0.1 milligram of glucose per litre.

Concentrations of peptone lower than 0.28 p.p.m. (which permitted growth of Bacterium coli) were found by Allen, Pasley and Pierce (1952) to be beneficial in maintaining the survival of Bacterium coli for several days. Ballantyne (1930) had previously observed that washing Bacterium typhosus with distilled water before addition to water or salt solution shortened its survival period. The survival of unwashed organisms was much longer because washing removed the nutritive material from the cells. It might well be that failure to wash thoroughly the organisms accounts for the undiminished survival during storage of Bacterium coli observed by Cook and Steele (1955). Similar effects of prolonging the survival time of organisms by the addition of organic materials, i.e., peptone, meat extract, serum, blood corpuscles extract, cystine, cysteine, were reported by Winslow and Brooke (1927), Zobell and Zobell (1932) and Davis (1940).

V Other Factors Influencing Bacterial Survival in Aqueous Fluids.

In addition to the above mentioned major factors influencing the viability of organisms in aqueous fluids, the effects of other factors, such as temperature, air, number

of bacteria per millilitre of suspension, are reported by various workers.

Ficker (1898) showed that in a dilute suspension of Vibrio cholerae in distilled water (1000 organisms per millilitre), nearly all the organisms died within 2 hours. When a more concentrated suspension was prepared (60 million organisms per millilitre) the bacteria remained viable for several months. Similar results were obtained by Platt (1935). None of the above mentioned authors realised a possible correlation between the concentration of organisms in suspension and the mortality rate. Such a correlation is proposed later in this publication.

The necessity of oxygen for the survival of Bacterium typhosus and the colon bacilli was shown by Whipple and Mayer (1906) who, demonstrated that these organisms remained viable for nearly 2 months in sterile tap water in the presence of air, but died within 4 days when the tubes were kept under hydrogen, nitrogen or carbon-dioxide.

Ballantyne (1930) demonstrated that the survival of Bacterium typhosus in water was longer at room temperature than at 37°C. Platt (1935) observed that Bacterium aerogenes survived longer than Bacterium coli at both 18°C and 37°C, but

at 0°C both survived equally well. Houston (1914) found that Bacterium typhosus lived in water for eight weeks at 0°C, 3 weeks at 18°C and only 1 week at 37°C. Clark and Lubs (1917) have said that "in cellular destruction the temperature is to be considered as an accelerating condition".

Thus, it appears that bacteria, generally speaking, die rapidly in distilled water while in water containing salts or organic matter they are able to survive undiminished in numbers for longer periods or perhaps even able to multiply.

SECTION II.

THE SCOPE OF THE PRESENT WORK.

THE SCOPE OF THE PRESENT WORK AND ITS RELATION TO PREVIOUS WORK ON THE BEHAVIOUR OF ORGANISMS IN TWO-PHASE SYSTEMS.

Studies of the viability of organisms in aqueous and oily systems have, hitherto, been confined to systems which were either known to be anhydrous, or which were regarded as being anhydrous. It has been shown, however, in the historical section that not all the oily systems examined were in fact anhydrous, but consisted of dispersions in oil of small droplets of water, or other aqueous fluids, in which living organisms were suspended.

In the present work an attempt has been made to inoculate dry oils with dried organisms and to study the fate of the organisms when the infected oil is brought into contact with an aqueous fluid. The only previous attempt to study the migration of organisms between two immiscible phases appears to have been undertaken by Mudd et al (1924 - 1927). The authors allowed oils to come into contact with a drop of aqueous suspension of the organisms under a cover-slip on a microscope slide. The preparations were observed under the microscope, and it was found that the organisms aggregated at the oil/water interface, and could only be displaced from the interface by the expenditure of mechanical energy. On displacement from

the interface the majority of the organisms passed back into the aqueous phase, only a very small proportion entering the oily phase. The result might well have been anticipated by the authors, since solid particles wetted with an aqueous fluid are normally immiscible with a hydrophobic phase.

Bullock and Booth (1953) floated over broth a volume of oil containing a spray-dried mixture of bacterial spores in peptone powder. Growth was obtained in the broth after incubation, indicating that one or more of the spores originally in the oil had migrated to the broth. The test for sterility described by the British Pharmacopoeia (1953) for oily preparations consists of floating a sample of the oil on broth and shaking the mixture at intervals during incubation. The test presumes that any organisms present in the oil will migrate to, and grow in the nutrient broth. The pharmacopoeial test is purely qualitative in nature. The present author has failed to find in the literature any reference relating to the quantitative study of the migration of organisms from one liquid phase to another. The present communication describes an attempt to study quantitatively the behaviour of organisms in both aqueous and oily phases separately, and when the two phases are in contact.

SECTION III.

E X P E R I M E N T A L

(A) INFECTION OF OILS WITH ORGANISMS FREEZE-DRIED FROM NUTRIENT BROTH.

In order that organisms can be dispersed in oils for viability studies, it is necessary to obtain them in a dry and viable condition. Drying may be accomplished by the simple process of storing the suspensions over a dehydrating agent in an evacuated desiccator, or by the process of freeze-drying. The latter method was selected for the present studies as being more expedient than simple desiccation, and because a product with an extremely low moisture content can be obtained.

I. The Preparation of dried organisms.

The medium used for suspending bacteria during freeze-drying is known to influence the mortality of the organisms during the freeze-drying process (Fry - 1951 and Proom - 1951). Experiments were, therefore, undertaken to ascertain a suitable suspending medium from which to dry Bacterium coli (N.C.T.C. No. 5933) and Bacillus subtilis (N.C.T.C. No.6346) used in the present investigation.

(a) The determination of the survival of Bacterium coli during freeze-drying.

The suitability of a suspending medium was ascertained by estimating the survival of the organisms during freeze-drying from the medium. This was estimated from viable counts performed by the roll-tube capillary-dropping-pipette technique before and after freeze-drying. The count before freeze-drying from a suspending fluid consisted of making suitable dilutions of the suspension of the organisms in quarter-strength Ringer's solution (Wilson's modified formula - 1922). Ten drops of the final dilution of the suspension were inoculated by means of a standard dropping-pipette into each of five tubes containing 2.5 millilitres of molten agar at 45°C. The inoculum was well mixed with the agar, the tubes were rolled under a cold water tap until the agar had solidified and then incubated in the inverted position at 37°C for 48 hours.

To ascertain the mortality of the organisms during the freeze-drying process, five tubes (from a single batch) of freeze-dried peptone powder containing Bacterium coli which resulted from the freeze-drying process were opened aseptically and the contents of the opened tubes were transferred to the five sterile tubes. This procedure was adopted because a similar procedure was necessary when transferring the freeze-dried powder to oils. Care was taken to transfer the powder as completely as possible from the freeze-dried tubes to the sterile tubes. Later results

(Page 112) showed that there was an insignificant loss during such transfer. The dried powder was resuspended in 100 drops of quarter-strength Ringer's solution and viable counts performed on the resultant suspension.

The sources of error in performing viable counts by the roll-tube capillary-dropping-pipette technique have been discussed by Berry and Michaels (1947) and by Bullock, Keepe and Rawlins (1949). The major errors are due to variations in the volume of bacterial suspension delivered by the pipettes, the non-homogeniety of the bacterial suspension, and to personal errors in counting the roll-tubes after incubation.

(i) The determination of variation in drop-volume of quarter-strength Ringer's solution delivered by the standard dropping-pipettes.

The standard dropping-pipettes used for measuring drops of bacterial suspension were described by Cook and Yousef (1953). The drop-volume of the quarter-strength Ringer's solution was calculated from the mean weight of 10 lots of 10 drops each, and from a knowledge of the specific gravity of the solution (Table 4).

TABLE 4

DROP VOLUME OF QUARTER-STRENGTH RINGER'S SOLUTION FROM STANDARD DROPPING-PIPETTE.

Weights of 10-drop lots.	Mean weight of 10-drop lots.	Mean weight of 1 drop.	Density of Ringer's Solution.	Volume of 1 drop.
0.173 g.				
0.173 g.				
0.171 g.	0.1742 g.	0.01742 g.		
0.172 g.				
0.175 g.			1.0043	0.0173 ml.
0.178 g.				
0.171 g.				
0.178 g.				
0.177 g.				
0.174 g.				

The uniformity of the weights of 10-drop lots from the same pipette, and the variation in weights due to the use of five different pipettes, is shown in Tables 5 and 6 respectively. When using the same pipette for determining the weight of 10-drop lots, the greatest and the mean deviations from the mean were 1.23 and 0.58 per cent respectively.

TABLE 5

WEIGHTS OF 10-DROP LOTS OF QUARTER-STRENGTH RINGER'S SOLUTION FROM ONE PIPETTE.

Weight of 10-drop lots of Ringer's Solution (X)	Mean weight of 10 drop lot ($\bar{X}$)	$(X - \bar{X})$	$(X - \bar{X})^2$
0.1692 G.		0.0015	0.00000225
0.1708 G.		0.0001	0.00000001
0.1696 G.		0.0011	0.00000121
0.1694 G.		0.0013	0.00000169
0.1700 G.		0.0007	0.00000049
0.1718 G.		0.0011	0.00000121
0.1692 G.		0.0015	0.00000225
0.1710 G.		0.0003	0.00000009
0.1686 G.		0.0021	0.00000441
0.1724 G.	0.1707 G.	0.0017	0.00000289
0.1712 G.		0.0005	0.00000025
0.1724 G.		0.0017	0.00000289
0.1712 G.		0.0005	0.00000025
0.1696 G.		0.0011	0.00000121
0.1692 G.		0.0015	0.00000225
0.1718 G.		0.0011	0.00000121
0.1714 G.		0.0007	0.00000049
0.1716 G.		0.0009	0.00000081
0.1702 G.		0.0005	0.00000025
0.1708 G.		0.0001	0.00000001

Standard deviation = 0.00114

Standard error of estimation. = 0.000255

TABLE 6

ANALYSIS OF VARIANCE of 20 WEIGHTS OF 10-DROP LOTS OF QUARTER-STRENGTH RINGER'S SOLUTION FROM FIVE DIFFERENT PIPETTES.

Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P.
(a) Between pipettes	0.001239	4	0.0003097	28.15	< 0.0001
(b) Within pipettes	0.001065	95	0.0000110		
Total	0.002304	99			

These values compare well with those obtained by Withell (1938) and by Cook and Yousef (1953). The between-pipette variance appears to be significant when compared with within-pipette variance. The variance ratio of 28.15 obtained in the present experiment is much lower than that obtained by either of the above workers. Using the total 100 weights of 10-drop lots from five different pipettes, the greatest and mean deviation from the mean were found to be 3.92 and 1.79 per cent respectively which also compare favourably with the values obtained by above workers. Hence it was decided to use these pipettes for measuring the bacterial suspensions in the present work.

(ii) The determination of personal error in counting the colonies after incubation.

When an incubated roll-tube is counted on successive occasions a variation in the counts obtained is observed. Wilson (1922), Berry and Michaels (1947) and Bullock, Keepe and Rawlins (1949) attributed this variation to the personal error in counting the colonies due to various factors enumerated by the last mentioned authors.

In the present experiments 10 drops of diluted suspension of Bacterium coli were added to a number of agar tubes, the tubes rolled and incubated in an inverted position at 37°C for 48 hours. The standard error of the mean of three counts from a series of roll tubes was found to be 1.79 (Table 7), which was regarded as satisfactory when compared with that obtained by the above-mentioned workers.

It was also observed that when the same roll-tubes were counted on three successive days after 24, 48 and 72 hours incubation, there was no real increase in the number of colonies on the second and third day. The difference in the counts observed was due to personal errors in counting and not to an increase in the number of colonies. It was, therefore, decided to count the tubes after 48 hours.

TABLE 7

ESTIMATION OF PERSONAL ERROR DUE TO COUNTING THE COLONIES IN INCUBATED ROLL-TUBES ON THREE OCCASIONS.

Expt No.	Counts (X) (N = 3)			Total S(X)	Mean $\bar{X}$	Corrected SS $S(X-\bar{X})^2 = S(X^2) - S\frac{^2X}{N}$			Variance (V) ($V = \frac{SS}{N}$)	S.E.(S). ($S = \sqrt{V}$).
	1st day	2nd day	3rd day			$S(X^2)$	$S\frac{^2X}{N}$	SS		
1	54	59	57	170	56.7	9646	9633	13	6.5	2.550
2	279	285	283	847	282.3	239155	239136	19	9.5	3.082
3	112	111	108	331	110.3	36529	36520	9	4.5	2.121
4	66	63	65	194	64.7	12550	12545	5	2.5	1.581
5	90	88	92	270	90.0	24308	24300	8	4.0	2.000
6	61	68	59	188	62.7	11820	11781	39	19.5	4.416
7	294	286	295	875	291.7	255257	255208	49	24.5	4.950
8	53	50	59	162	54.0	8790	8748	42	21.0	4.583
9	73	70	69	212	70.7	14990	14981	9	4.5	2.121
10	266	261	262	789	263	207521	207507	14	7.0	2.646
11	126	132	128	386	128.7	49684	49665	19	9.5	3.082
12	57	66	59	182	60.7	11086	11008	78	39	6.245
13	96	92	93	281	93.7	26329	26320	9	4.5	2.121
14	53	53	55	161	53.7	8643	8640	3	1.5	1.225
15	80	78	79	237	79.0	18725	18723	2	1.0	1.000
16	60	61	62	183	61.0	11165	11163	2	1.0	1.000
17	113	110	111	334	111.3	37190	37185	5	2.5	1.581
18	82	80	77	239	79.7	19053	19040	13	6.5	2.550
19	61	60	57	178	59.3	10570	10561	9	4.5	2.121
20	64	62	64	190	63.3	12036	12033	3	1.5	1.225
	Total			6412				350		

Total sum of square for 60 observations = 350.

Sum of square for count variation $SS/N = 350/40 = 8.75$.

Standard deviation for count variation = $\sqrt{8.75} = 2.958$.

∴ Standard error of a mean of 3 counts = $8.75/3 = 1.709$.

(iii) The determination of the over-all error in the viable count technique.

Thornton (1922) suggested that the medium, on which organisms are grown, should be sensitive enough to enable any viable organism to grow when incubated at its optimal temperature. He suggested that if 20-replicate roll-tubes were prepared from a single suspension of organisms and the colonies counted after incubation, the index of dispersion (χ^2) for the colonies would provide a measure of suitability of the medium. The index of dispersion examines rather more than the suitability of the medium and includes an assessment of the whole of the technique involved in making 20-replicate counts on a bacterial suspension.

In the experiments recorded, the values of χ^2 computed from the counted colonies were 12.03 and 11.7 (Tables 8 and 9). The probability of obtaining such values for 19 degrees of freedom is between 0.8 and 0.9, indicating that one would expect to obtain χ^2 of the observed value in between 8 and 9 out of 10 experiments, if the over-all variation observed was due to random sampling errors. Thus the technique of performing a viable count on a single suspension may be regarded as satisfactory.

(b) The survival of Bacterium coli during freeze-drying.

(i) Freeze-dried from a broth containing sodium chloride.

TABLE 8

GOODNESS OF FIT OF χ^2 VALUES FOR 20 TUBES USED IN THE ESTIMATION OR THE SUITABILITY OF THE DROPPING-PIPPETTE-ROLL-TUBE TECHNIQUE.

Counts (X)	Mean Count ($\overline{X}$)	Deviations from Mean Count ($X - \overline{X}$)	$(X - \overline{X})^2$	χ^2	P
56		6	36		
57		5	25		
57		5	25		
69		7	49		
56		6	36		
57		5	25		
57		5	25		
70		8	64		
56		6	36	$746/62$	0.9-
68	62	6	36	$= 12.03$	0.8
68		6	36		
58		4	16		
56		6	36		
70		8	64		
70		8	64		
66		4	16		
69		7	49		
56		6	36		
56		6	36		
68		6	36		

TABLE 9

GOODNESS OF FIT OF χ^2 VALUES FOR 20 TUBES USED IN THE ESTIMATION OF THE SUITABILITY OF THE DROPPING-PIPETTE-ROLL-TUBE TECHNIQUE.

Counts (X)	Mean Count ($\bar{X}$)	Deviations from Mean Count ($X-\bar{X}$)	$(X - \bar{X})^2$	χ^2	P.
119		9	81		
101		9	81		
119		9	81		
101		9	81		
103		7	49		
105		5	25		
119		9	81	$\frac{1288}{110}$	0.9 to
118		8	64		
101	110	9	81	= 11.70	0.8
102		8	64		
104		6	36		
120		10	100		
118		8	64		
104		6	36		
102		8	64		
119		9	81		
108		8	64		
105		5	25		
103		7	49		

The effect of the suspending medium on the mortality of the organisms during the freeze-drying process has been studied by Fry (1951) and others. In the preliminary experiments Bacterium coli was freeze-dried from a fluid medium containing 3% $^{W}/_{V}$ "Oxoid" peptone, 1% $^{W}/_{V}$ Lab Lemco and 0.5% $^{W}/_{V}$ sodium chloride. The mortality during freeze-drying was very high, there being only about 2 per cent survivors (Table 10). The high mortality observed can be attributed to a marked increase in the osmotic pressure of the fluid which resulted from concentration of the sodium chloride during freeze-drying. The percentage survival was too low to provide suitable counts when added to the oil and it was, therefore, decided to try to freeze-dry the organisms from a medium containing no sodium chloride.

(ii) Freeze-dried from a broth without sodium chloride.

When Bacterium coli was freeze-dried from a broth of the above composition but containing no sodium chloride the organisms were found to survive satisfactorily and thus confirmed the belief that the high mortality previously observed was due to the high osmotic pressure of the fluid during drying. In three different experiments between 26.1 and 28.15 per cent organisms were found to survive the freeze-drying process (Table 11). This survival was regarded very satisfactory when compared with the survival reported for

TABLE 10

THE SURVIVAL OF BACTERIUM COLI DURING FREEZE-DRYING FROM PEPTONE BROTH CONTAINING SODIUM CHLORIDE.

	Sample Tube	Counts of 5-replicate tubes.					Mean Count	Log. Mean Count.	Log. Dilution factor.	No. of organisms per ml. of original suspension.	Percentage Survival
Before freeze-drying		156	165	170	159	158	161.6	2.2073	8.3541	36,533,720,000	
After freeze-drying	No.1.	56	50	49	57	52	57.28	1.7578	7.1075	733,768,500	2.00
	No.2.	58	52	60	62	60					
	No.3.	54	60	63	55	62					
	No.4.	57	64	55	56	63					
	No.5.	62	64	55	60	56					

TABLE 11

THE SURVIVAL OF BACTERIUM COLI DURING FREEZE-DRYING FROM PEPTONE BROTH WITHOUT SODIUM CHLORIDE.

Experiment.		Sample Tubes	Counts of 5-replicate tubes.					Mean Count.	Log. Mean Count.	Log. Dilution factor.	No. of organisms per ml. of original suspension.	Percentage Survival.
I	Before freeze-drying		146	138	142	152	161	151.8	2.1812	8.3541	34,318,185,000	
	After freeze-drying	No.1.	24	22	26	27	27					
		No.2.	28	21	28	28	24					
		No.3.	22	20	24	26	20	23.4	1.3692	8.5832	8,962,384,900	26.1
		No.4.	22	20	19	25	22					
		No.5.	22	20	24	26	21					

TABLE 11 Cont'd.

Experiment.		Sample Tube	Counts of 5-replicate tubes.					Mean Count.	Log. Mean Count.	Log. Dilution factor.	No. of organisms per ml. of original suspension.	Percentage Survival.
II	Before freeze-drying		127	140	138	134	126	133.00	2.1239	8.3541	30,067,975,000	
	After freeze-drying	No.1.	166	154	170	152	170					
		No.2.	160	181	168	169	178					
		No.3.	165	179	163	160	174	165.84	2.2196	7.7080	8,465,714,584	28.15
		No.4.	162	158	169	150	169					
		No.5.	160	174	160	162	173					

TABLE 11 Cont'd

Experiment.		Sample Tube	Counts of 5-replicate tubes.					Mean Count.	Log. Mean Count.	Log. Dilution factor.	No. of organisms per ml. of original suspension.	Percentage Survival.
III	Before freeze-drying		117	127	115	116	126	120.2	2.0799	8.3541	27,174,215,000	
	After freeze-drying	No.1.	39	36	38	33	39					
		No.2.	40	39	32	36	35					
		No.3.	36	38	36	44	38	37.64	1.5757	8.2821	7,210,581,127	26.53
		No.4.	48	39	40	36	39					
		No.5.	40	32	36	38	34					

vegetative organisms during spray-drying by Bullock, Keepe and Rawlins (1949) and by Bullock and Keepe (1951).

An Analysis of Variance of the counts indicates that the mortality observed during freeze drying was consistent from tube to tube and that the organisms were uniformly distributed between each of the tubes (Table 12).

TABLE 12

ANALYSIS OF VARIANCE OF QUINTUPLICATE ROLL-TUBING OF FIVE SAMPLE TUBES OF THE SAME FREEZE-DRIED PEPTONE POWDER CONTAINING BACTERIUM COLI.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
I	(a) Between samples	70	4	17.5	2.121	0.1-0.2
	(b) Within Samples	165	20	8.25		
	Total	235	24			
II	(a) Between Samples	321	4	80.25	1.477	>0.2
	(b) Within Samples	1087	20	54.35		
	Total	1408	24			
III	(a) Between Samples	64	4	16	1.06	>0.2
	(b) Within Samples	302	20	15.1		
	Total	366	24			

(c) The survival during storage of Bacterium coli in freeze-dried peptone powder.

Before the freeze-dried powder could be added to the oils for viability studies it was necessary to determine the survival of the organisms during storage in the dried powder.

The survival of the organisms during storage under nitrogen at room temperature was determined by opening a tube of the freeze-dried material at specified intervals during 100 days, reconstituting the suspension and performing viable counts on the reconstituted suspension (Table 13 and Fig.III). The percentage survival was calculated in relation to the number of organisms surviving the freeze-drying process. The organisms died off gradually during storage; after 100 days approximately 40 per cent of the organisms which survived the freeze-drying process were still viable.

The rate of death of Bacterium coli observed during storage in freeze-dried peptone powder was much less than that observed by Bullock, Keepe and Rawlins (1949) for Bacterium lactis aerogenes stored in spray-dried peptone powder or for Streptococcus faecalis stored under similar condition (Bullock and Keepe - 1951). The death-rate was, however, greater than that observed by the former workers when Bacillus subtilis spores were similarly stored. The

TABLE 13

THE SURVIVAL DURING STORAGE OF BACTERIUM COLI FREEZE-DRIED FROM PEPTONE BROTH.

Duration of Storage.	Counts of 5-replicate tubes.					Mean Count.	Log. Mean Count.	Log. Dilution Factor.	No. of organisms per ml. of suspension.	Percentage Survival
0 hours	72	66	75	75	67	71.0	1.8513	7.4075	1817,508,160	
24 hours	70	66	74	69	74	70.6	1.8488	7.4075	1805,138,220	99.32
192 hours (8 days)	67	60	68	65	60	64.0	1.8062	7.4075	1636,385,920	90.13
360 hours (15 days)	62	56	56	63	65	60.4	1.7810	7.4075	1544,339,210	84.97
720 hours (30 days)	55	50	58	57	51	54.2	1.7340	7.4075	1385,814,330	76.25
1440 hours (60 days)	86	81	90	82	90	85.8	1.9335	7.1075	1099,446,790	60.49
2400 hours (100 days)	55	63	55	62	54	57.8	1.7619	7.1075	741,487,370	40.8

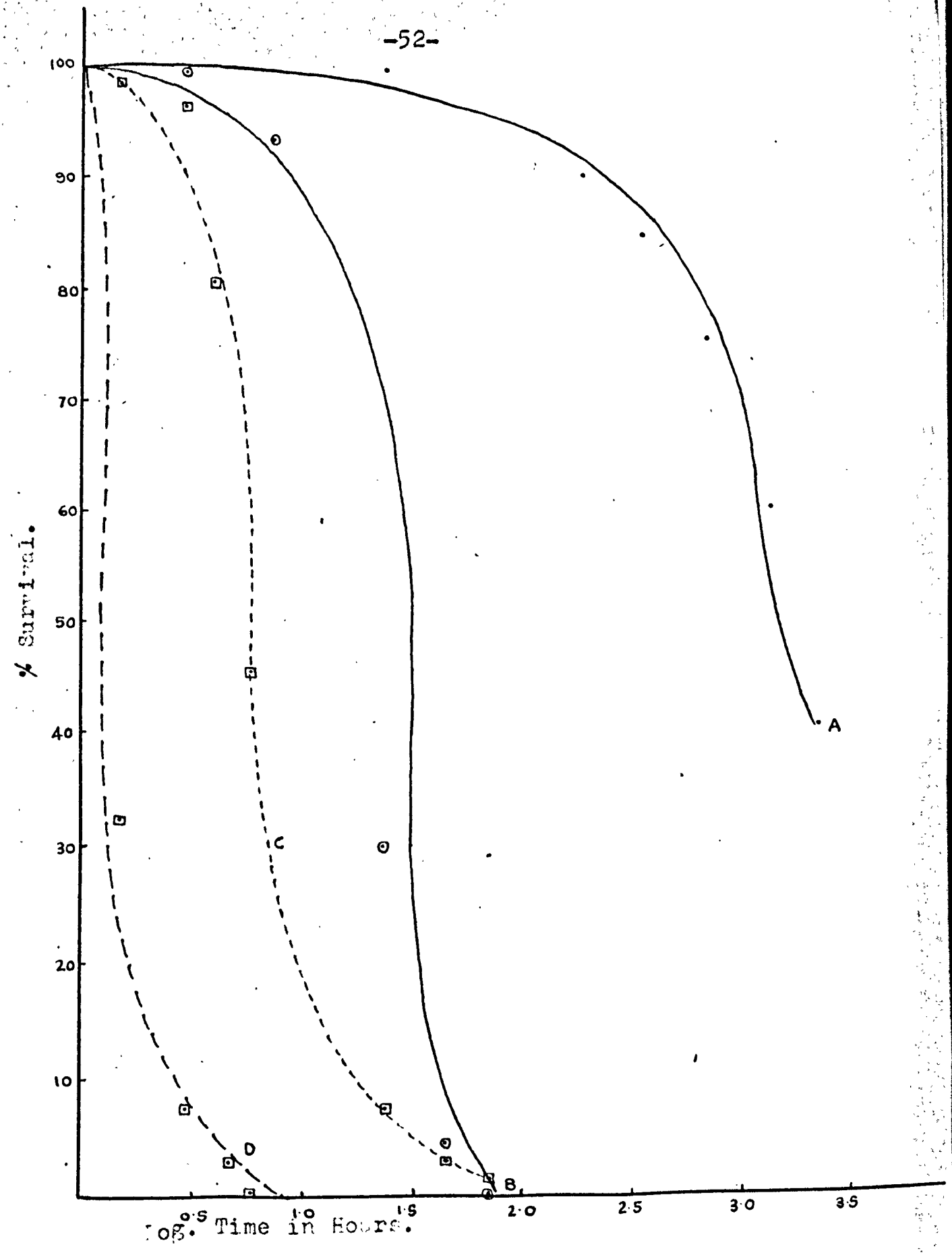


Fig. III The Survival of Bacterium coli

A:- Freeze-dried peptone material.
B:- Freeze-dried peptone material in light liquid paraffin.
C:- Water freeze-dried organisms.
D:- Water freeze-dried organisms in light liquid paraffin.

strain of Bacterium coli used in the present study is known to be comparatively resistant to lethal agents, and thus it appears that the mortality of the organisms when stored in the dried condition is related in part to their normal resistance to unfavourable environments.

II The Infection of Oils by Dry Organisms.

Fairhall and Bates (1920) appear to be the first workers to attempt to introduce dry and free spores into fixed oils. Later Bullock and Keepe (1951) infected various fixed and mineral oils with spray-dried peptone or stearin powders containing Streptococcus faecalis or spores of Bacillus subtilis. They triturated their powders in a sterile mortar with oil and then diluted the suspension with a further quantity of the same sterile oil. In the present experiments the quantity of the freeze-dried powder used for infecting the oils was very small and thus the technique used by Bullock and Keepe was suitably modified as described below.

A number of tubes containing Bacterium coli in freeze-dried peptone powder were opened and the contents transferred aseptically to a sterile tube containing glass beads and 25 millilitres of sterile light liquid paraffin. The tube was vigorously shaken to break up and disperse the adherent mass of powder as much as possible. After shaking

for about five to ten minutes some particles were still visible and, hence the tube was allowed to stand for five minutes to allow the larger particles to sediment. The supernatant oil containing the dispersed Bacterium coli in peptone powder was pipetted off into another sterile tube and shaken to promote homogeneity, after which it was diluted with a further quantity of sterile light liquid paraffin.

III Viable Counts of Organisms Introduced into Oils.

The technique described by Bullock and Keepe (1951) for counting bacteria in oils is the most reliable and accurate so far developed. It consists of dissolving a sample of the infected oil in a volatile solvent, centrifuging to sediment the organisms and pipetting off the supernatant. The precipitated organisms are twice washed with further small quantities of organic solvent, and after the second washing, the last traces of organic solvent are removed by applying reduced pressure; finally the organisms remaining behind in the centrifuge tube are resuspended in sterile Ringer's solution and viable counts performed on the aqueous suspension.

(a) The mortality of Bacterium coli in petroleum ether (b.pt. 40° - 60° C).

Bullock and Keepe (1951) have shown that the majority of organic solvents which are suitable for dissolving fixed oils have a bactericidal action on organisms suspended in them. It is, therefore, necessary to ascertain the precise mortality of an organism when submitted to the action of an organic solvent.

Petroleum ether (b.pt. 40° - 60°C) was selected for the present studies as it is a good solvent for light liquid paraffin and other fixed oils, and is sufficiently volatile to be removed by means of reduced pressure.

The bactericidal activity of petroleum ether (b.pt. 40° - 60°C) against Bacterium coli was determined by transferring the contents of five tubes containing freeze-dried peptone powder with Bacterium coli to five sterile tubes and performing viable counts on them. The contents of a further five tubes from the same batch of freeze-dried peptone powder containing Bacterium coli were transferred to five sterile centrifuge tubes and three millilitres of petroleum ether were added to each of them. The powder was mixed with the solvent by gently shaking the tubes, care being taken not to touch the cotton wool-plug. After about 15 minutes the tubes were centrifuged at 3500 r.p.m. for two minutes. After exactly 20 minutes most of the supernatant solvent was pipetted off. The period of contact was

standardised at 20 minutes as preliminary experiments had indicated this to be the time required to complete the counting technique for organisms in the oils (described below). The last traces of petroleum ether were evaporated off under reduced pressure for 20 minutes. After complete removal of the solvent the remaining powder was resuspended in 100 drops of Ringer's solution and viable counts performed (Table 14).

Table 14 shows that petroleum ether (b.pt. 40° - 60°C) had no bactericidal effect on Bacterium coli in freeze-dried peptone powder. This result was unexpected, especially as Bullock and Keepe (1951) had found that petroleum ether and all the other organic solvents used by them killed vegetative organisms readily. The result did, however, reaffirm the suitability of the solvent for the specis of Bacterium coli used in the present experiments.

(b) The enumeration of viable organisms in oils.

In the experiments in the present work five samples of infected oil were placed in centrifuge tubes, the oil dissolved in the solvent, and the mixture centrifuged. The remaining deposit was twice washed, the traces of solvent removed under reduced pressure, the residue resuspended in aqueous fluid and the viable counts performed.

TABLE 14

THE SURVIVAL OF BACTERIUM COLI FREEZE-DRIED FROM PEPTONE BROTH AFTER TREATMENT FOR 20 MINUTES WITH PETROLEUM ETHER (B.PT 40° - 60°C).

Experiment		Sample tubes	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of Ringer's solution	Percentage Survival
I	Freeze-dried powder containing Bacterium Coli	No.1.	50	53	50	51	59	52.8	1.7226	8.2821	10,114,736,544	
		No.2.	57	56	52	51	54					
		No.3.	50	52	48	54	52					
		No.4.	57	59	53	52	55					
		No.5.	50	47	53	52	53					
	Freeze-dried powder containing Bacterium Coli treated for 20 minutes with petroleum ether.	No.1.	51	52	56	50	53	52.16	1.7173	8.2821	9,992,133,677	98.79
		No.2.	55	57	50	50	58					
		No.3.	51	52	60	53	56					
		No.4.	50	48	55	49	48					
		No.5.	52	50	46	52	47					

TABLE 14 Cont'd

Experiment		Sample Tubes	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of Ringer's solution	Percentage Survival.
II	Freeze-dried powder containing Bacterium Coli	No.1.	98	94	91	99	95	95.08	1.9781	7.7080	4,854,180,091	
		No.2.	102	96	99	93	98					
		No.3.	92	96	90	93	99					
		No.4.	96	91	97	98	93					
		No.5.	90	90	94	95	98					
	Freeze-dried powder containing Bacterium Coli treated for 20 minutes with petroleum ether	No.1.	93	97	98	101	92	98.02	1.9913	7.7080	5,004,482,007	103.1
		No.2 .	94	99	98	90	92					
		No.3.	101	106	99	104	98					
		No.4.	98	92	90	89	90					
		No.5.	96	94	97	98	100					

TABLE 14 Cont'd.

Experiment		Sample Tubes	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of Ringer's solution	Percentage Survival
III	Freeze-dried powder containing Bacterium Coli	No.1.	67	72	74	69	76	76.0	1.8808	7.7080	3,880,076,640	
		No.2.	80	72	73	78	81					
		No.3.	79	76	83	85	79					
		No.4.	74	79	81	73	76					
		No.5.	80	72	71	73	77					
	Freeze-dried powder containing Bacterium Coli treated for 20 minutes with petroleum ether.	No.1.	84	80	76	77	81	77.0	1.8865	7.7080	3,931,130,280	101.3
		No.2.	79	76	75	81	74					
		No.3.	69	74	77	76	76					
		No.4.	80	76	84	80	78					
		No.5.	76	72	68	78	78					

(i.) The determination of the drop-volume of light liquid paraffin from a standard dropping-pipette.

The drop-volume of Ringer's solution has been determined previously (Table 4). The drop-volume from a standard dropping-pipette is influenced by the density and viscosity of the fluid. In order to know the exact amount of the sample of oil used, the drop-volume of light liquid paraffin was determined.

Ten lots of 50-drops each of light liquid paraffin were collected from a capillary-dropping pipette and weighed. The density of the light liquid paraffin was determined and the drop-volume calculated. (Table 15). Wilson (1922) and Withell (1938) have stated that the presence of organisms in a fluid does not materially affect the drop-volume of that fluid, and it was, therefore, assumed in the following experiments that the drop-volume of the infected light liquid paraffin was not significantly different from that determined for pure light liquid paraffin.

(ii.) The enumeration of Bacterium coli in light liquid paraffin.

Fifty drops of light liquid paraffin infected with

TABLE 15

DROP-VOLUME OF LIGHT LIQUID PARAFFIN DELIVERED BY A STANDARD DROPPING CAPILLARY-PIPETTE.

Weight of 50-drop lots.	Mean weight of 50-drop lots	Mean weight of 1 drop.	Density of light liquid Paraffin.	Volume of 1 drop.
0.4446 g.				
0.4390 g.				
0.4440 g.				
0.4420 g.	0.4435 g.	0.00887 g.	0.852	0.0104
0.4414 g.				
0.4412 g.				
0.4438 g.				
0.4442 g.				
0.4510 g.				
0.4438 g.				

freeze-dried peptone powder containing *Bacterium coli* were added to each of five sterile centrifuge tubes. Three millilitres of petroleum ether (b.pt. 40° to 60°C) were added to each of the tubes and the contents thoroughly mixed by gentle shaking. The tubes were immediately centrifuged at 3500 r.p.m. for 2 minutes and the supernatant solvent pipetted off. The deposits in each of the tubes were washed

twice with similar quantities of fresh solvent. After exactly 20 minutes most of the supernatant solvent was pipetted off from the tubes and the last traces of it were evaporated by applying reduced pressure for 20 minutes. The residues were resuspended in separate portions of 100 drops of Ringer's solution and viable counts performed in quintuplicate on each suspension (Table 16). An Analysis of Variance of the counts obtained from the five samples of oil gave a probability of between 0.1 and 0.2 which signified that the between sample variation was not significantly different from the within sample variation and, therefore, that the viable counts obtained on different samples of the infected oil were uniform (Table 17), from which it was inferred that the organisms were uniformly distributed in the oil.

(c) The Survival of Bacterium coli (in freeze-dried peptone powder) during storage in light liquid paraffin.

The viability of the organisms in light liquid paraffin during storage at room temperature (25°C) was determined by performing viable counts on the samples of infected oil immediately after preparation of the suspension and at specified intervals during 72 hours (Table 18, Fig.III). During the period of storage the suspension was constantly rotated about the horizontal axis so as to prevent the organisms from settling down.

TABLE 16

QUINTUPLICATE COUNTS OF FIVE SAMPLES OF THE SAME LIGHT LIQUID PARAFFIN CONTAINING BACTERIUM COLI FREEZE-DRIED FROM PEPTONE BROTH.

Experiment	50-drops samples of oil	Volume of Ringer's solution added	Counts of 5-replicate tubes					Total Count per Sample
I	No.1.	1.73 ml.	66	74	70	76	76	362
	No.2.	1.73 ml.	78	70	76	78	80	382
	No.3.	1.73 ml.	71	78	80	75	70	374
	No.4.	1.73 ml.	72	66	66	75	75	354
	No.5.	1.73 ml.	70	80	70	72	70	362
II	No.1.	1.73 ml.	53	59	50	60	57	279
	No.2.	1.73 ml.	68	59	59	64	59	309
	No.3.	1.73 ml.	66	60	58	60	62	306
	No.4.	1.73 ml.	59	52	62	52	57	282
	No.5.	1.73 ml.	60	66	56	63	57	302

The viable counts indicated that after 72 hours storage fewer than 0.5 per cent organisms remained viable, whereas in the freeze-dried peptone powder there was nil mortality during the same period of storage under nitrogen.

TABLE 17

ANALYSIS OF VARIANCE OF QUINTUPLICATE ROLL-TUBING OF FIVE SAMPLES OF THE SAME LIGHT LIQUID PARAFFIN CONTAINING PEPTONE POWDER WITH BACTERIUM COLI.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
I	(a) Between samples	99	4	24.75	1.349	>0.2
	(b) Within samples	367	20	18.35		
	Total	466	24			
II	(a) Between samples	158	4	39.5	2.461	0.1 - 0.05
	(b) Within samples	321	20	16.05		
	Total	479	24			

It can thus be concluded that the light liquid paraffin was bactericidal to Bacterium coli in the freeze-dried peptone powder. This finding is at variance with that reported by Bullock and Keepe (1951) who found that Streptococci faecalis survived in light liquid paraffin for six months although there was an initial fall in the number of viable organisms. Bullock and Keepe concluded from their results that vegetative

organisms died no more rapidly in the liquid paraffin than in the spray-dried peptone powder itself. The present results show on the contrary, that the light liquid paraffin does exert a definite bactericidal action on Bacterium coli in freeze-dried peptone powder.

TABLE 18

THE SURVIVAL IN LIGHT LIQUID PARAFFIN OF BACTERIUM COLI FREEZE-DRIED FROM PEPTONE BROTH.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
I	0 Hour	No.1.	66	74	70	76	76	73.36	1.8655	5.1196	9,665,473	
		No.2.	78	70	76	78	80					
		No.3.	71	78	80	75	70					
		No.4.	72	66	66	75	75					
		No.5.	70	80	70	72	70					
	3 Hours	No.1.	62	72	70	71	64	73.52	1.8664	5.1196	9,686,554	100.22
		No.2.	78	74	70	82	74					
		No.3.	80	70	70	80	74					
		No.4.	78	68	76	77	69					
		No.5.	75	76	70	80	79					

TABLE 18 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
I	6 Hours	No.1.	62	72	70	71	66	72.72	1.8616	5.1196	9,581,150	99.13
		No.2.	76	73	68	77	75					
		No.3.	70	78	68	79	78					
		No.4.	76	69	77	68	77					
		No.5.	77	75	69	79	78					
	24 Hours	No.1.	224	219	202	206	209	208.2	2.3185	4.3470	4,628,286	47.88
		No.2.	219	200	200	203	215					
		No.3.	198	196	196	214	210					
		No.4.	207	206	225	218	205					
		No.5.	204	217	198	213	201					

TABLE 18 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival.
I	48 Hours	No.1.	51	60	60	60	54	57.28	1.7580	4.0468	637,984	6.60
		No.2.	59	64	54	60	54					
		No.3.	60	50	59	51	58					
		No.4.	57	67	62	56	57					
		No.5.	59	51	61	60	51					
	72 Hours	No.1.	80	75	84	86	78	82.0	1.9138	2.7551	46682	0.48
		No.2.	90	80	79	80	89					
		No.3.	80	78	75	84	84					
		No.4.	91	82	80	88	80					
		No.5.	74	85	81	83	74					

TABLE 18 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. or organisms per ml. of oil	Percentage Survival
II	0 Hour	No.1.	53	59	50	60	57	59.12	1.7717	5.1196	7,789,296	
		No.2.	68	59	59	64	59					
		No.3.	66	60	58	60	62					
		No.4.	59	52	62	52	57					
		No.5.	60	66	56	63	57					
	3 Hours	No.1.	51	59	51	49	59	59.00	1.7709	5.1196	7,773,486	99.80
		No.2.	63	60	60	70	61					
		No.3.	57	67	63	56	57					
		No.4.	63	64	55	54	58					
		No.5.	60	56	65	62	55					

TABLE 18 Cont'd

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	6 Hours	No.1.	49	59	51	52	58	55.4	1.7435	5.1196	7,299,171	93.70
		No.2.	54	61	57	51	62					
		No.3.	62	52	52	55	60					
		No.4.	59	50	50	51	58					
		No.5.	53	56	50	59	61					
	24 Hours	No.1.	113	108	104	117	104	105.28	2.0220	4.3470	2,340,374	30.04
		No.2.	98	108	96	106	108					
		No.3.	107	108	97	97	100					
		No.4.	100	108	105	111	98					
		No.5.	112	111	100	112	104					

TABLE 18 Cont'd

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes	Mean Count	Log. Mean Count	Log. Dilution Factor	No. or organisms per ml. of oil	Percentage Survival
II	48 Hours	No.1.	65 65 66 55 58	60.32	1.7799	3.7605	347,503	4.46
		No.2.	50 60 60 58 50					
		No.3.	60 64 66 53 59					
		No.4.	64 56 66 56 55					
		No.5.	63 69 60 58 66					
	72 Hours	No.1.	90 80 79 89 80	84.64	1.9276	2.4362	23,106	0.29
		No.2.	91 79 80 89 86					
		No.3.	80 91 86 82 91					
		No.4.	92 80 81 89 83					
		No.5.	88 80 81 80 89					

(B.) THE MIGRATION OF BACTERIA FROM AN OILY TO AN AQUEOUS PHASE.

The British Pharmacopoeia (1953) contains a test for sterility of oily suspensions or solutions which involves floating a sample of the oil on the surface of nutrient broth and shaking the two phases together. The test presumes that any organism present in the oil will migrate into the broth and produce growth on incubation. Bullock and Booth (1953) performed a test similar to that of the British Pharmacopoeia and demonstrated that when an oil containing spores of Bacillus subtilis in spray-dried peptone powder was floated on sterile broth, the organisms eventually grew in the broth indicating that they had migrated from the oily to the aqueous phase. Both the pharmacopoeial test and that of Bullock and Booth (1953) are qualitative tests, whereas the experiments described in the present work sought to determine quantitatively the migration of organisms from an oily to an aqueous phase.

I. The Survival of Organisms in Aqueous Fluids.

The experiments envisaged consisted of floating a volume of an infected oil on the surface of a sterile aqueous fluid and performing viable counts at specified

intervals on both the oily and aqueous phases. It has already been shown in previous experiments (Table 18) that Bacterium coli die in light liquid paraffin. Hence, for proper assessment of death during migration from the oily to the aqueous phase it was necessary to use an aqueous fluid that was without any bactericidal effects during the experimental period.

(a) The survival of Bacterium coli in aqueous fluids.

Wilson(1922) and others have demonstrated the bactericidal action of distilled waters and the protective action on the organisms of quarter-strength Ringer's solution. (Wilson's modified formula - 1922). Experiments were, therefore, undertaken to confirm that quarter-strength Ringer's solution was a more suitable suspending fluid than distilled water for the strain of organism used in the present experiments.

A suspension of Bacterium coli was prepared from a 24-hour agar culture. The organisms were thoroughly washed by centrifuging in three separate portions of distilled water. This procedure was adopted to prevent the carry over of traces of nutritive material, since Allan, Paisley and Pierce (1952) have shown that 0.28 p.p.m. of

organic matter in distilled water is sufficient to promote bacterial growth. Viable counts were performed on suspensions of Bacterium coli in :-

(i) distilled water from a metal still (Table 19),

(ii) distilled water from a heavily tinned still with an all glass condensing system (Table 20),

(iii) quarter-strength Ringer's solution prepared from distilled water (ii) (Table 21 and Fig.IV).

After only 8 hours storage at room temperature there were no survivors in the distilled water obtained from a metal still. On the other hand, in the distilled water from the tinned still, and in the Ringer's solution prepared from it, there was nil kill after a similar period of storage. After 48 hours storage in distilled water from the tinned still 73 per cent of organisms were still viable, whereas in the Ringer's solution after similar storage there were 92 per cent survivors. These results confirmed the findings of Wilson (1922) and others that Ringer's solution was a better suspending fluid for Bacterium coli than distilled water.

(b) The relationship between the survival of Bacterium coli in aqueous fluids and the density of the bacterial suspension.

TABLE 20

THE SURVIVAL OF BACTERIUM COLI IN DISTILLED WATER FROM A TINNED STILL WITH ALL GLASS CONDENSOR.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of suspension	Percentage Survival
0 Hour	118	119	113	106	120	115.2	2.0615	8.0573	13148,218,368	.
1 Hour	116	113	114	116	106	111.0	2.0453	8.0573	12668,856,240	96.35
2 Hours	115	104	112	103	103	107.4	2.0311	8.0573	12257,974,416	93.23
4 Hours	95	102	92	105	96	98.0	1.9912	8.0573	11185,116,320	85.07
8 Hours	87	96	86	95	86	90.0	1.9542	8.0573	10272,045,600	78.13
24 Hours	93	84	81	92	85	87.0	1.9395	8.0573	9929,644,080	75.52
48 Hours	87	89	89	80	80	85.0	1.9294	8.0573	9701,376,400	73.79

TABLE 21

THE SURVIVAL OF BACTERIUM COLI IN QUARTER-STRENGTH RINGER'S SOLUTION PREPARED IN DISTILLED WATER FROM TINNED STILL WITH ALL GLASS CONDENSER.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of Organisms per ml. of suspension	Percentage Survival
0 Hour	62	68	60	66	60	63.2	1.8007	8.0573	7213,258,688	
1 Hour	65	66	60	66	69	63.2	1.8007	8.0573	7213,258,688	100.00
2 Hours	60	64	62	66	69	64.2	1.8075	8.0573	7327,392,528	101.60
4 Hours	56	68	66	68	60	63.6	1.8035	8.0573	7258,912,224	100.60
8 Hours	63	60	66	60	67	63.2	1.8007	8.0573	7213,258,688	100.00
24 Hours	123	123	121	109	120	119.2	2.0763	7.7636	6917,354,800	95.90
48 Hours	116	118	114	102	116	113.2	2.0539	7.7636	6569,165,800	91.07

TABLE 21 Cont'd.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension.	Percentage Survival
96 Hours	98	98	106	94	106	100.2	2.0008	7.7636	5826,362,600	80.77
120 Hours	92	89	80	87	94	88.4	1.9465	7.7636	5129,984,600	71.12
144 Hours	80	89	78	82	77	81.2	1.9096	7.7636	4712,157,800	65.33
168 Hours	70	78	68	81	76	74.6	1.8727	7.7636	4329,149,900	59.87

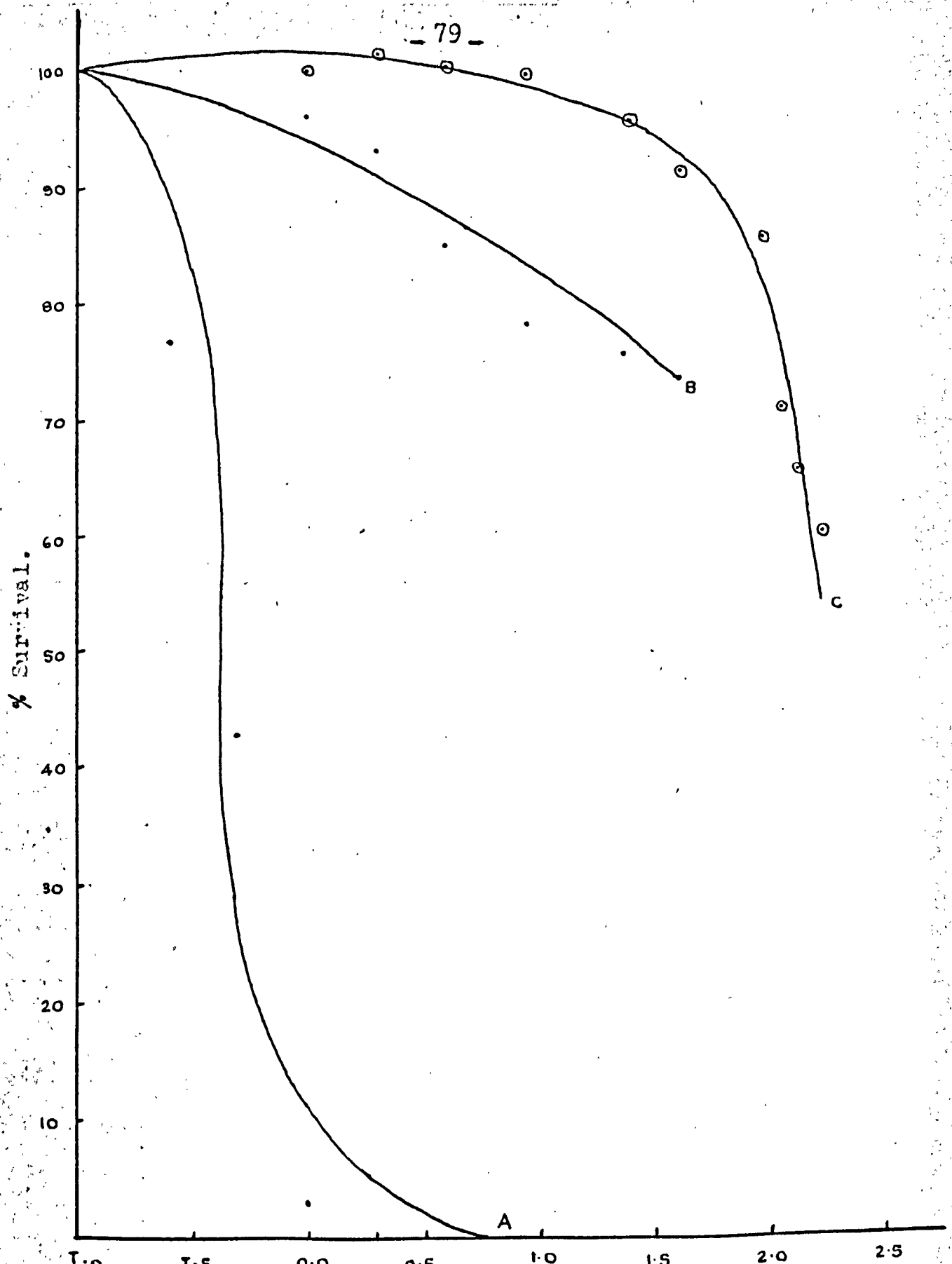


Fig. IV The Survival of Bacterium coli in Aqueous Fluids.
A:- Distilled water from a metallic still.
B:- Distilled water from tinned still.
C:- Quarter-strength Ringer's solution.

During the course of the above described experiments on the survival of Bacterium coli in aqueous fluids, it was observed that there appeared to be some relationship between the initial concentration of the organisms in the suspending fluids and the survival rate in these fluids. No record has been found in the literature recording quantitative results showing the relationship between the density of the inoculum and the survival rate in distilled water. Ficker (1898), however, stated that Vibrio cholerae in a heavy inoculum in distilled water survived much longer than did a light inoculum. Experiments recorded in Tables 22 to 29 and Fig. V indicate clearly that when Bacterium coli were suspended in distilled water (ii), the death-rate was much greater with a small inoculum than with a large inoculum, whereas in the quarter-strength Ringer's solution the size of inoculum had no effect on the death-rate.

The envisaged migration experiments outlined above involved floating infected oil on the surface of an aqueous fluid, and estimating the rate of migration of the organisms from the oil to the lower aqueous phase by performing viable counts at intervals on both phases. A true assessment of the rate of migration of viable organisms is only possible if there is neither death nor multiplication of the organisms in the aqueous phase. Quarter-strength Ringer's solution most nearly fitted this requirement and it was,

TABLE 22

THE SURVIVAL OF BACTERIUM COLI IN DISTILLED WATER FROM TINNED STILL.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of suspension	Percentage Survival.
0 Hour	118	119	113	106	120	115.2	2.0615	8.0573	13148,218,368	
1 Hour	116	113	114	106	106	111.0	2.0453	8.0573	12668,856,240	96.35
2 Hours	115	104	112	103	103	107.4	2.0311	8.0573	12257,974,416	93.23
4 Hours	95	102	92	105	96	98.0	1.9912	8.0573	11185,116,320	85.07
8 Hours	87	96	86	95	86	90.0	1.9542	8.0573	10272,045,600	78.13
24 Hours	93	84	81	92	85	87.0	1.9395	8.0573	9929,644,080	75.52
48 Hours	87	89	89	80	80	85.0	1.9294	8.0573	9701,376,400	73.79

TABLE 23

THE SURVIVAL OF BACTERIUM COLI IN DISTILLED WATER FROM TINNED STILL.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor.	Number of organisms per ml. of suspension	Percentage Survival
0 Hour	139	125	130	133	138	133.0	2.1239	6.8885	1028,808,200	
1 Hour	130	132	122	120	131	127.0	2.1038	6.8885	982,395,800	95.49
2 Hours	131	120	118	122	117	121.6	2.0828	6.8885	940,624,640	91.43
4 Hours	119	107	115	106	108	111.0	2.0453	6.8885	858,629,400	83.46
8 Hours	93	107	98	108	104	102.0	2.0086	6.8885	789,010,800	76.70
24 Hours	102	90	91	103	89	95.0	1.9777	6.8885	734,863,000	71.43
48 Hours	76	86	85	86	77	82.0	1.9138	6.8885	634,302,800	61.66

TABLE 24

THE SURVIVAL OF BACTERIUM COLI IN DISTILLED WATER FROM TINNED STILL.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival.
0 Hour	279	257	260	261	278	267.0	2.4265	0.7634	1549	
1 Hour	184	168	183	171	172	175.6	2.2445	0.7634	1018	65.70
2 Hours	132	143	143	138	130	137.2	2.1373	0.7634	795	51.39
4 Hours	81	96	88	95	85	89.0	1.9494	0.7634	516	33.32
8 Hours	55	51	49	54	46	51.0	1.7076	0.7634	295	19.05
24 Hours	14	19	13	13	19	15.6	1.1931	0.7634	90	5.82
48 Hours	0	0	0	0	0	0	0	0.7634	0	0

TABLE 25

THE RELATIONSHIP BETWEEN THE SURVIVAL OF BACTERIUM COLI AND THEIR CONCENTRATION IN DISTILLED WATER FROM TINNED STILL.

Concentration of Bacterium coli in distilled water	Percentage survival after storage						
	0 Hour	1 Hour	2 Hours	4 Hours	8 Hours	24 Hours	48 Hours
1300 x 10^6 approximately	100.00	96.35	93.23	85.07	78.13	75.52	73.79
1000 x 10^9 approximately	100.00	95.49	91.43	83.46	76.70	71.43	61.66
1.5 x 10^3 approximately	100.00	65.70	51.39	33.32	19.05	5.82	0.00

TABLE 26

THE SURVIVAL OF BACTERIUM COLI IN QUARTER-STRENGTH RINGER'S SOLUTION.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival.
0 Hour	62	68	60	66	60	63.2	1.8007	8.0573	7213,258,688	
1 Hour	65	66	60	66	59	63.2	1.8007	8.0573	7213,258,688	100.00
2 Hours	60	64	62	66	69	64.2	1.8075	8.0573	7327,392,528	101.60
4 Hours	56	68	66	68	60	63.6	1.8035	8.0573	7258,912,224	100.60
8 Hours	63	60	66	60	67	63.2	1.8007	8.0573	7213,258,688	100.00
24 Hours	123	123	121	109	120	119.2	2.0763	7.7636	6917,354,800	95.90
48 Hours	116	118	114	102	116	113.2	2.0539	7.7636	6569,165,800	91.07

TABLE 26 Cont'd.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilu-tion Factor	No. of organisms per ml. of suspension	Percentage Survival
96 Hours	98	98	106	94	106	100.2	2.0008	7.7636	5826,362,600	80.77
120 Hours	92	89	80	87	94	88.4	1.9465	7.7636	5129,984,600	71.12
144 Hours	80	89	78	82	77	81.2	1.9096	7.7636	4712,157,800	65.33
168 Hours	70	78	68	81	76	74.6	1.8727	7.7636	4329,149,900	59.87

TABLE 27

THE SURVIVAL OF BACTERIUM COLI IN QUARTER-STRENGTH RINGER'S SOLUTION.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor.	No. of organisms per ml. of suspension	Percentage survival
0 Hour	73	72	78	69	67	71.2	1.8525	6.2878	138,194,928	
1 Hour	76	69	70	76	64	71.0	1.8513	6.2878	137,806,740	99.72
2 Hours	69	73	76	64	75	71.4	1.8537	6.2878	138,533,116	100.28
4 Hours	76	76	70	63	68	70.6	1.8488	6.2878	137,030,364	99.36
8 Hours	68	74	66	73	75	71.2	1.8525	6.2878	138,194,928	100.00
24 Hours	73	68	69	73	60	68.6	1.8363	6.2878	133,148,484	96.35
48 Hours	68	63	60	69	70	65.0	1.8129	6.2878	126,161,100	91.30

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilu-tion Factor	No. of organisms per ml. of suspension	Percentage Survival
96 Hours	140	152	144	140	139	143.0	2.1553	5.8918	111,475,364	80.67
120 Hours	120	134	128	120	130	126.4	2.1018	5.8918	98,534,486	71.30
144 Hours	113	114	121	119	109	115.6	2.0640	5.8918	90,115,748	65.21
168 Hours	114	101	115	116	101	109.4	2.0391	5.8918	85,282,660	61.71

TABLE 28

THE SURVIVAL OF BACTERIUM COLI IN QUARTER-STRENGTH RINGER'S SOLUTION.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
0 Hour	156	157	140	147	156	151.2	2.1796	0.7634	877	
1 Hour	145	159	148	153	159	152.8	2.1840	0.7634	886	101.06
2 Hours	141	158	146	149	156	150.0	2.1761	0.7634	870	99.22
4 Hours	148	161	153	148	145	151.0	2.1790	0.7634	876	99.88
8 Hours	150	163	146	148	154	152.2	2.1824	0.7634	882	100.66
24 Hours	140	140	148	156	142	145.2	2.1620	0.7634	842	96.03
48 Hours	144	126	137	130	133	134.0	2.1271	0.7634	777	88.63

TABLE 28 Cont'd

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilu-tion Factor	No. of organisms per ml. of suspension	Percentage Survival
96 Hours	119	112	130	126	122	121.8	2.0856	0.7634	706	80.56
120 Hours	102	114	101	109	111	107.4	2.0311	0.7634	623	71.06
144 Hours	106	94	99	103	91	98.6	1.9939	0.7634	571	65.24
168 Hours	83	96	89	95	99	92.4	1.9657	0.7634	536	61.12

TABLE 29

THE RELATIONSHIP BETWEEN THE SURVIVAL OF BACTERIUM COLI AND THEIR CONCENTRATION IN QUARTER-STRENGTH RINGER'S SOLUTION.

Concentration of Bacterium coli in quarter-strength Ringer's solution.	Percentage survival after storage.										
	0 Hour	1 Hour	2 Hours	4 Hours	8 Hours	24 Hours	48 Hours	96 Hours	120 Hours	144 Hours	168 Hours
7200 x 10^6 approximately	100.00	100.00	101.60	100.60	100.00	95.90	91.70	80.77	71.12	65.33	59.87
130 x 10^6 approximately	100.00	99.72	100.28	99.36	100.00	96.35	91.30	80.67	71.30	65.21	61.71
8.7 x 10^2 approximately	100.00	101.06	99.22	99.88	106.66	96.03	88.63	80.56	71.06	65.24	61.12

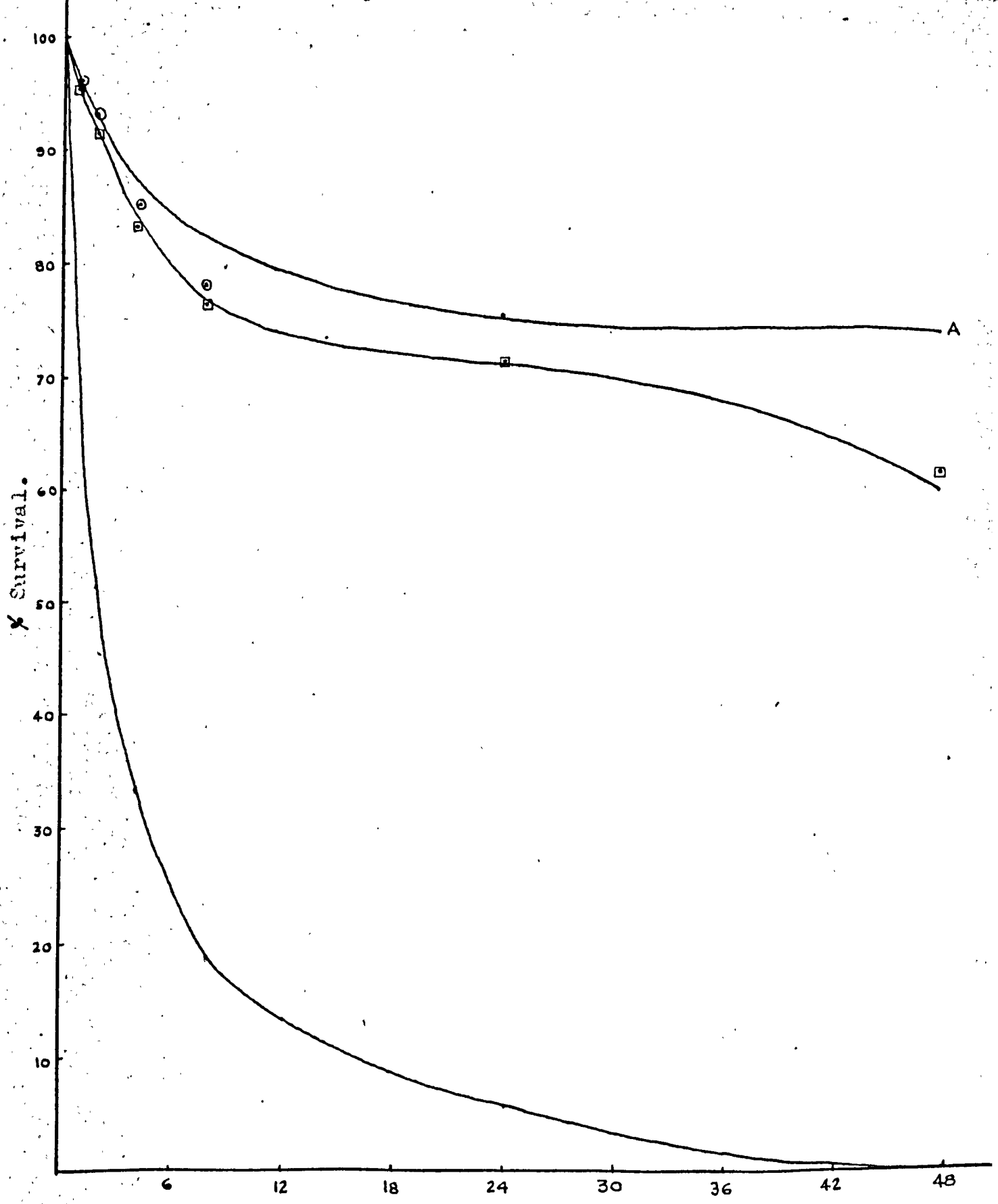


Fig. V The Survival of Different Concentrations of Bacterium coli in Distilled Water from Tinned Still.

A:- 13000 x 10^6 approximately.
B:- 1000 x 10^6 approximately.
C:- 1·5 x 10^3 approximately.

therefore, selected as the aqueous medium in all experiments as the slow death-rate of the organisms in the Ringer's solution was found to be independent of the inoculum size.

II The Migration of Bacterium coli from Light Liquid Paraffin (in Peptone Freeze-dried Powder) to Ringer's Solution.

Viable counts performed on samples of a suspension of oil containing Bacterium coli in freeze-dried peptone showed that equal volumes of oil contained equal number of viable organisms (Table 17) suggesting that the organisms were uniformly dispersed in the oil. In the migration experiments the infected oily suspension was to be floated on an aqueous layer in a series of separators. In order that the rate of migration of the organisms from the oily to the aqueous phase could be studied, it was necessary to know first whether the rate of sedimentation of the organisms through the oil and their subsequent passage into the Ringer's solution, was the same from separator to separator.

To study this, light liquid paraffin was infected with freeze-dried peptone powder containing Bacterium coli. Fifteen millilitres of the infected light liquid paraffin containing a known number of organisms was floated over ten millilitres of sterile Ringer's solution in each of a

series of ten cylindrical glass-stoppered separators of uniform dimensions and 40 millilitres capacity. The two phases were allowed to remain in contact for 15 minutes after which the oil was pipetted off from five separators without disturbing the interface. Ringer's solution was also collected from the same five separators. Viable counts were performed on a sample of the oils from each of the separators after shaking the oils thoroughly and on the Ringer's solutions collected from each of these separators. Further, viable counts were performed on samples of the oils, and Ringer's solutions removed from the remaining five separators after 30 minutes contact between the two phases (Table 30).

Samples of the infected oil which had been stored in a container rotated about a horizontal axis gave reproducible counts (Table 17). After floating on the Ringer's solution for 15 minutes equal volumes of oil taken from each of the five separators no longer contained equal numbers of viable organisms. If the rate of sedimentation had been uniform in the five separators, the counts on each of the samples should still have been equal, within the limits of experimental errors. The fact that counts on the oils taken from the five separators were significantly different (Table 31) indicated that the rate of sedimentation of the organisms varied significantly from separator to separator.

TABLE 30

VARIATION OF COUNTS OF BACTERIUM COLI IN OIL AND RINGER'S SOLUTION FROM DIFFERENT SEPARATORS.

Experiment		Duration of Contact	Counts from different separators				
			1	2	3	4	5
I	Oil	15 Minutes	47	32	100	71	52
			39	39	91	69	48
			50	34	87	60	57
			42	38	92	64	49
			41	34	88	66	55
		30 Minutes	0	0	0	0	0
			0	0	0	0	0
			0	0	0	0	0
			0	0	0	0	0
			0	0	0	0	0
	Ringer's Solution	15 Minutes	155	213	79	97	107
			143	227	90	102	109
			144	228	79	92	118
			155	216	88	100	109
			150	22[illegible]	82	93	115
		30 Minutes	158	170	160	172	167
			163	158	164	162	156
			152	157	151	164	155
			163	168	164	170	159
			163	160	153	165	166

Number of organisms initially in the oil :- 16,941,000.

Number of organisms in Ringer's solution after 30 minutes :- 16,216,000.

TABLE 30 Cont'd.

Experiment		Duration of Contact	Counts from different separators				
			1	2	3	4	5
II	Oil	15 Minutes	134	235	197	270	106
			148	249	185	286	97
			139	250	192	288	104
			142	240	183	274	105
			146	236	190	282	98
		30 Minutes	0	0	0	0	0
			0	0	0	0	0
			0	0	0	0	0
			0	0	0	0	0
			0	0	0	0	0
	Ringer's Solution	15 Minutes	155	213	79	97	107
			143	227	90	102	109
			144	228	79	92	118
			155	216	88	100	109
			150	222	82	93	115
		30 Minutes	158	170	160	172	167
			163	158	164	162	156
			152	157	151	164	155
			163	168	164	170	159
			163	160	153	165	166

Number of organisms initially in the oil :- 20,349,900

Number of organisms in Ringer's solution after 30 Minutes :-

21,158,000.

TABLE 31

ANALYSIS OF VARIANCE ON FIVE SAMPLES OF OIL FROM FIVE SEPARATORS AFTER 15 MINUTES SEDIMENTATION.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
I	(a) Between Separators	9694	4	2423.5	136.1	<0.001
	(b) Within Separators	356	20	17.8		
	Totals	10050	24			
II	(a) Between Separators	104331	4	26082.75	684	<0.001
	(b) Within Separators	762	20	38.1		
	Totals	105093	24			

After the infected oil had been in contact with the Ringer's solution for 30 minutes, the samples of oils taken from the five separators contained no viable organisms. Thus, all the organisms which initially infected the oil had sedimented into the Ringer's solution in between 15 and 30 minutes (Table 30). Some of the organisms can be assumed to have sedimented in that time through a distance equal to the height of the oil column, i.e., 4 centimetres. If the density and

viscosity of the oil are known, and if it can be assumed that dry Bacterium coli has a density of approximately 1.0 (because under normal conditions an organism is in osmotic equilibrium with water the density of which is 1.0), and if Stoke's law can be applied to the system, it is possible to estimate the probable limits of the minimal sizes of the bacterial particles sedimenting through the oil. Strictly Stoke's law applies only to spherical particles and when applied to non-spherical particles can give only a very approximate indication of the rate of sedimentation.

If in the equation

$$v = \frac{2}{9} \cdot \frac{(p1-p2)r^2g}{\eta}$$

p1 = density of the particle

p2 = density of the liquid

r = radius of the particle

g = gravitational force

η = viscosity of the liquid

v = terminal velocity of fall

then the smallest particle which could have sedimented through a four centimetre column of oil in between 15 and 30 minutes would have a diameter of between approximately 1160 and 820 microns.

Bacterium coli is reported by Topley and Wilson (1948) and by Bergey (1948) to have a maximal dimension of

approximately 0.6 x 3.0 microns. No Bacterium coli could possibly reach such a dimension as 820 microns and the presence of particles having such large dimensions is only reconcilable with the organisms being present in very large aggregates which could be produced, either by the organisms adhering to one another in the oil, or by their being embedded in particles of peptone.

It is conceivable that particles having very much larger dimensions than calculated are present in the oil. That is, the oil contained particles which varied appreciably in size. The rate of sedimentation of particles of such varied size would also vary; that is, the particles in the oil would sediment at a non-uniform rate.

An Analysis of Variance on the counts performed on the Ringer's solutions collected from the separators after 15 minutes contact with the infected oil is given in Table 32. The "between separator" variance was significantly greater than the "within separator" variance. This provided confirmation that the organisms passed from the oil to the Ringer's solution in each of the five separators at significantly different rates, and therefore consisted of aggregates of varying sizes. After 30 minutes contact between the two phases, all the organisms initially in the oil were found to be in the Ringer's solutions. This

was confirmation of the very rapid rate of sedimentation of the organisms through the oil, and supported the inference that they were present in the oil in exceedingly large aggregates (Table 33).

TABLE 32

ANALYSIS OF VARIANCE ON FIVE SAMPLES OF RINGER'S SOLUTION FROM FIVE SEPARATORS AFTER 15 MINUTES SEDIMENTATION.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
I	(a) Between separators	61279	4	15319.75	532	<0.001
	(b) Within separators	575	20	28.75		
	Total.	61854	24			
II	(a) Between separators	43164	4	10791	520	<0.001
	(b) Within separators	435	20	21.75		
	Total.	43599	24			

The above described experiments indicated that the organisms were not present in the oil as free living individual cells but probably embedded in particles of freeze-dried peptone. The behaviour in oils of such embedded organisms

would undoubtedly be different from that of organisms not so embedded in peptone. They would, for example, never come into direct contact with the oil and thus any bactericidal activity possessed by an oil would not be apparent from a consideration of the death-rate of embedded organisms.

TABLE 33

ANALYSIS OF VARIANCE OF FIVE SAMPLES OF RINGER'S SOLUTION FROM FIVE SEPARATORS AFTER 30 MINUTES SEDIMENTATION.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
I	(a) Between separators	202	4	50.5	1.730	0.1-0.2
	(b) Within separators	584	20	29.2		
	Total	786	24			
II	(a) Between separators	192	4	48.0	2.554	0.05-0.1
	(b) Within separators	376	20	18.8		
	Total.	568	24			

C. INFECTION OF OILS BY ORGANISMS FREEZE-DRIED FROM DISTILLED WATER.

The experiments described above led to the conclusion that when bacteria are freeze-dried from a nutrient medium, the resultant freeze-dried material consists of particles of the dried medium in which the organisms are embedded. The behaviour of such embedded organisms is likely to be very different from that of freely suspended organisms. It was, therefore, decided to attempt to freeze-dry Bacterium coli from distilled water to produce dried organisms free from any adherent protective material.

I. The Survival of Bacterium coli during Freeze-drying from Distilled Water.

The mortality of Bacterium coli during freeze-drying from suspensions in distilled water was determined by the method described previously (page 34). In two freeze-drying experiments 2.06 and 2.56 per cent of the organisms survived the freeze-drying process (Table 34). An Analysis of Variance of the counts obtained in these experiments is given in Table 35. The probability of obtaining such a variance ratio was greater than 0.2 which suggested that the mortality observed during freeze-drying was consistent and that the

TABLE 34

THE SURVIVAL OF BACTERIUM COLI DURING FREEZE-DRYING FROM DISTILLED WATER.

Experiment		Sample Tube	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
	Before Freeze-drying		56	53	50	58	59	55.2	1.7419	8.4154	14,367,574,880	
I	After Freeze-drying	No.1.	114	104	110	106	108					
		No.2.	116	109	110	108	101					
		No.3.	104	111	100	109	106	106.8	2.0286	6.4434	296,552,521	2.06
		No.4.	110	100	114	106	104					
		No.5.	102	100	109	99	110					
	Before Freeze-drying		240	228	222	240	220	228.0	2.3579	8.1176	29,906,452,200	
II	After Freeze-drying	No.1.	141	130	130	139	137					
		No.2.	144	134	149	145	135					
		No.3.	141	130	126	142	136	137.8	2.1393	6.7441	746,626,707	2.56
		No.4.	145	143	134	130	140					
		No.5.	146	143	134	130	141					

freeze-dried organisms are distributed uniformly in each of the tubes.

TABLE 35

ANALYSIS OF VARIANCE OF QUINTUPLICATE ROLL-TUBING OF FIVE SAMPLE TUBES FROM THE SAME FREEZE-DRIED MATERIAL CONTAINING BACTERIUM COLI.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P.
I	(a) Between tubes of freeze-dried material	75	4	18.75	1.271	>0.2
	(b) Within suspension	295	20	14.75		
	Total	370	24			
II	(a) Between tubes of freeze-dried material	248	4	62.0	1.897	0.1+ 0.2
	(b) Within suspension	654	20	32.7		
	Total	902	24			

The survival of Bacterium coli was less than 10 per cent of that obtained when the same organism was freeze-dried from a peptone solution. This indicated that the peptone and other nutritive materials present in the earlier experiments

protected the organisms from the lethal effects of the freeze-drying process. Such protective effects of nutritive materials on the mortality of the organisms during freeze-drying have been reported by Proom (1951), Fry (1951) and others.

II. The Survival during Storage of Dry "Uncoated" Bacterium coli.

Bacterium coli which had survived freeze-drying from distilled water died off rapidly on storage under nitrogen at room temperature. In two experiments only 42.13 and 45.24 per cent respectively of the organisms which survived the freeze-drying process were still viable after 6 hours storage (Table 36 and Fig.III), whereas approximately the same percentage survived for 100 days when they were freeze-dried from peptone solution (Table 13 and Fig.III).

Thus the suspending medium can have a preservative influence on the organisms both during the freeze-drying process and during subsequent storage.

The reason Bacterium coli dried from distilled water cannot be used satisfactorily is not that there are still 55 per cent survivors after 6 hours storage but because after 48 hours storage only about 3.00 per cent of those organisms surviving the freeze-drying process are still alive. This

TABLE 36

THE SURVIVAL DURING STORAGE OF BACTERIUM COLI FREEZE-DRIED FROM DISTILLED WATER.

Experiment	Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of suspension	Percentage Survival of organisms surviving freeze-drying
I	0 Hour	62	55	57	63	54	58.2	1.7649	6.4434	161,603,400	
	1½ Hours	61	53	57	62	54	57.4	1.7589	6.4434	159,382,580	98.63
	3 Hours	60	51	57	61	52	56.2	1.7497	6.4434	156,050,540	96.56
	4½ Hours	92	86	97	98	92	93.0	1.9685	6.1459	130,158,987	80.54
	6 Hours	52	50	59	50	51	52.4	1.7193	6.1459	73,336,891	45.38
	24 Hours	273	283	263	270	276	273.0	2.4362	4.6623	12,544,350	7.76
	48 Hours	107	118	111	107	117	112.0	2.0492	4.6623	5,146,400	3.18
	72 Hours	56	51	59	59	52	55.4	1.7435	4.6623	2,545,630	1.57

TABLE 36 Cont'd

Experiment	Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of suspension	Percentage Survival of organisms surviving Freeze-drying
	0 Hour	81	74	70	78	71	74.8	1.8739	6.7441	415,051,362	
	1½ Hours	82	70	72	70	78	74.4	1.8716	6.7441	412,831,836	99.46
	3 Hours	78	71	68	67	76	72.0	1.8573	6.7441	399,514,680	96.26
	4½ Hours	65	60	54	55	68	60.4	1.7810	6.7441	335,148,426	80.75
II	6 Hours	60	71	58	66	60	63.0	1.7993	6.4434	174,932,730	42.15
	24 Hours	106	90	97	102	92	97.4	1.9886	5.4499	27,450,144	6.61
	48 Hours	91	82	90	75	75	82.6	1.9170	5.1523	11,732,751	2.82
	72 Hours	52	49	57	50	48	51.2	1.7093	5.1523	7,272,601	1.75

is equivalent to about 0.06 per cent of the original number present before freeze-drying and is too low a number for viability studies in the oils where the death-rate might be considerably accentuated. Because of the rapid death-rate on storage, Bacterium coli when employed was used immediately after freeze-drying. This entailed the preparation of a fresh batch of freeze-dried material for each experiment.

Thus, the decision was taken to resort to a sporing organism (Bacillus subtilis) which might be more resistant to both the freeze-drying process and subsequent storage.

III. The Preparation of Dried Spores of Bacillus subtilis.

Spores of Bacillus subtilis (N.C.T.C.No.6346) were obtained by growing the organism on peptone agar slopes at 37°C for 48 hours, washing off the growth and heating the thoroughly washed suspension in distilled water at 90°C for 20 minutes to kill the vegetative cells. The spores were maintained by freeze-drying from distilled water since the experiments with Bacterium coli had indicated that the presence of nutritive materials influences the survival of the organisms.

A number of agar tubes infected with ten drops of suspensions of Bacillus subtilis spores were rolled and incubated for 24 hours. The standard-error due to counting the colonies of Bacillus subtilis, in the incubated roll-tubes,

on three different occasions was 1.719. This compared favourably with that obtained for the same organism by Bullock, Keepe and Rawlins (1949).

There was no increase in the daily count of the colonies after incubation for three successive days, but there was a marked increase in spreading and enlargement of the colonies after more than 24 hours incubation. The spreading tended to produce confluent growth and increased the difficulty of counting the colonies. It was, therefore, decided to count Bacillus subtilis colonies after 24 hours incubation.

(a) The survival of spores of Bacillus subtilis during freeze-drying from distilled water.

The survival of Bacillus subtilis spores recorded during freeze-drying from distilled water on two occasions is shown to be between 97.0 and 101.5 per cent. (Table 37). These deviations from 100 per cent survival can be attributed to random errors. The survival obtained was better than the 88 per cent survival obtained by Bullock, Keepe and Rawlins (1949) during spray-drying of spores of Bacillus subtilis from peptone suspension. It was, thus, concluded that the freeze-drying process had no deleterious effect on the strain of Bacillus subtilis spores used.

TABLE 37

THE SURVIVAL OF BACILLUS SUBTILIS SPORES DURING FREEZE-DRYING.

Experiment		Sample Tube	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor.	No. of organisms per ml. of original suspension	Percentage Survival
I	Before freeze-drying		182	191	196	180	180	185.8	2.2691	8.4154	48360,423,470	
	After freeze-drying	No.1.	56	63	55	60	65					
		No.2.	54	63	59	54	55					
		No.3.	64	54	60	55	64	58.12	1.7643	8.9240	48801,734,000	100.91
		No.4.	60	50	56	50	60					
		No.5.	60	54	61	55	66					
II	Before freeze-drying		59	66	64	60	71	64.00	1.8062	8.7156	33253,809,280	

TABLE 37 Cont'd.

Experiment		Sample Tube	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of original suspension	Percentage Survival
II	After freeze-drying	No.1.	92	96	85	99	88	96.28	1.9836	8.5250	32252,729,000	97.0
		No.2.	92	96	101	90	99					
		No.3.	99	98	106	92	103					
		No.4.	94	110	94	101	94					
		No.5.	90	98	94	98	98					

The "between tubes of freeze-dried material" variance was not significantly different from "the within tubes of freeze-dried material" variance. The probability of obtaining the variance ratio observed in both experiments was about 0.2 which suggested that the variation in the number of viable organisms in each tube at the end of the freeze-drying process can be attributed to random errors. In other words, if any tube of freeze-dried material is selected randomly from a batch, the number of viable organisms contained in the tube will not be significantly different from that in any other tube of the same batch selected in a similar manner (Table 38).

(b) The survival during storage of the freeze-dried spores of Bacillus subtilis.

The survival of the freeze-dried spores of Bacillus subtilis during storage under nitrogen at room temperature was calculated with reference to the number of spores surviving the freeze-drying process (Table 39 and Fig. VI). There was no reduction in the number of viable dried spores after 100 days storage. Bullock, Keene and Rawlins (1949) obtained similar results when their spores in spray-dried peptone powder were stored for six months at room temperature over phosphorus pentoxide. Thus it can be concluded that, whereas vegetative organisms such as Bacterium coli tend to die very

rapidly in the dried condition, the dried spores of Bacillus subtilis survive for a very long period and are, therefore, more suitable for studies in oils.

TABLE 38

ANALYSIS OF VARIANCE OF QUINTUPLICATE ROLL-TUBING OF FIVE SAMPLE TUBES FROM THE SAME FREEZE-DRIED MATERIAL CONTAINING BACILLUS SUBTILIS SPORES.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
I	(a) Between tubes of freeze-dried material	73	4	18.25	1.127	>0.2
	(b) Within suspension	324	20	16.2		
	Total	397	24			
II	(a) Between tubes of freeze-dried material	178	4	44.5	1.537	>0.2
	(b) Within suspension	579	20	28.95		
	Total	757	24			

TABLE 39

THE SURVIVAL DURING STORAGE OF BACILLUS SUBTILIS SPORES FREEZE-DRIED FROM DISTILLED WATER.

Duration of Storage	Sample Tube	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage survival of organisms surviving freeze-drying.
0 Hours	No.1.	90	98	95	101	90	96.28	1.9836	8.5250	322,52,720,000	
	No.2.	90	100	98	90	102					
	No.3.	102	98	104	92	96					
	No.4.	96	105	98	94	92					
	No.5.	90	102	90	96	102					
24 Hours	No.1.	97	100	98	89	100	96.8	1.9859	8.5250	32431,637,200	100.6
96 Hours	No.1.	96	98	104	90	94	96.4	1.9841	8.5250	32297,622,134	100.1
8 days	No.1.	96	96	88	97	86	92.6	1.9666	8.5250	31024,479,352	96.2
15 days	No.1.	84	94	88	86	94	89.2	1.9504	8.5250	29885,351,601	92.7

TABLE 39 Cont'd.

Duration of Storage	Sample Tubes	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension.	Percentage Survival of organisms surviving freeze-drying
30 days	No.1.	90	90	99	99	95	94.6	1.9759	8.5250	31694,554,500	98.3
60 days	No.1.	100	103	92	91	96	96.4	1.9841	8.5250	32297,622,134	100.1
100 days	No.1.	97	101	90	89	103	96.0	1.9823	8.5250	321,636,07,104	99.72

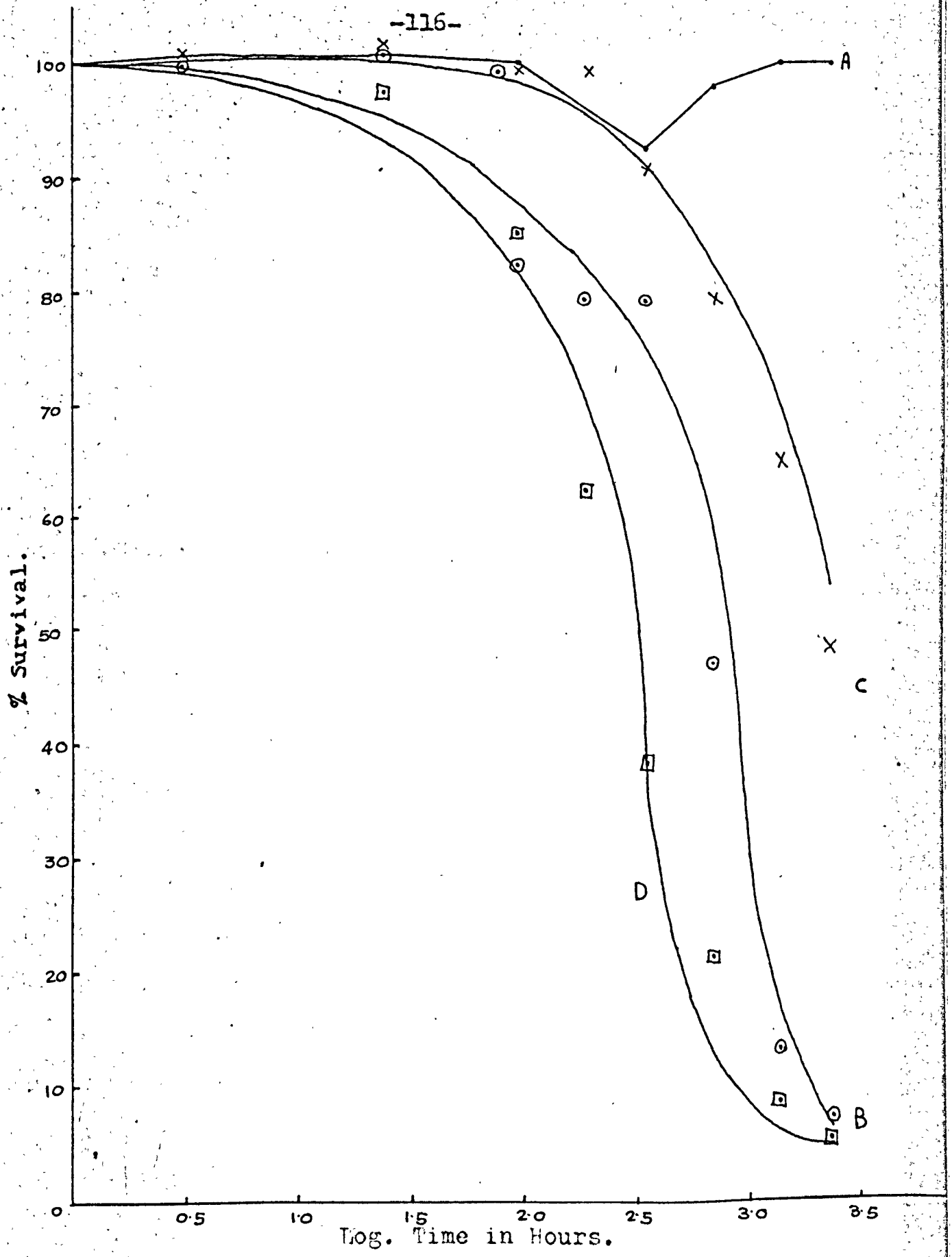


Fig.VI The Survival of Spores of Bacillus subtilis.
A:- Dry "uncoated" organisms.
B:- Dry "uncoated" organisms in light liquid paraffin.
C:- Dry "uncoated" organisms in a mixture of liquid paraffins.
D:- Dry "uncoated" organisms in arachis oil.

IV. The Infection of Light Liquid Paraffin by Dry "Uncoated" Bacterium coli and spores of Bacillus subtilis.

When the freeze-dried cells of Bacterium coli or the spores of Bacillus subtilis were added to light liquid paraffin in a tube and the tube shaken vigorously, a cloudy suspension was produced with very few visible clumps. The suspension, thus formed, was lightly centrifuged at 1000 r.p.m. for 1 minute to deposit very large particles and to produce a fine and more uniform suspension.

(a) The survival of dried "uncoated" Bacterium coli and spores of Bacillus subtilis in petroleum ether (b.pt. 40° - 60° C).

It has been shown previously (page 105) that peptone and other nutritive materials protect Bacterium coli both during freeze-drying and on storage in the freeze-dried state. The coating of nutritive materials on freeze-dried organisms can also be expected to influence their survival in fat solvents, such as petroleum ether (b.pt. 40° - 60°C), used in the technique for the enumeration of viable organisms in oil.

The number of dry "uncoated" Bacterium coli or Spores of Bacillus subtilis surviving for 20 minutes in petroleum ether (b.pt. 40° - 60°C) is recorded in Tables

TABLE 40

THE SURVIVAL OF DRY "UNCOATED" BACTERIUM COLI AFTER TREATMENT WITH PETROLEUM ETHER (B.Pt. 40° - 60°C) FOR 20 MINUTES.

Experiment		Sample Tube.	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
I	Dry "Uncoated" Bacterium coli	No.1.	200	203	213	200	200	198.84	2.2985	5.5619	72,528,878	
		No.2.	190	187	200	201	200					
		No.3	190	204	204	189	202					
		No.4.	196	198	196	180	194					
		No.5.	198	210	209	197	210					
	Dry "uncoated" Bacterium coli after treatment with petroleum ether for 20 minutes	No.1.	120	132	132	120	122	122.76	2.1229	4.8660	9,016,967	12.43
		No.2.	120	132	129	121	132					
		No.3.	110	119	112	120	109					
		No.4.	120	131	118	120	131					
		No.5.	119	132	118	119	131					

TABLE 40 Cont'd.

Experiment		Sample Tube	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of suspension	Percentage Survival
II	Dry "uncoated" Bacterium coli	No.1.	126	114	126	127	116	119.04	2.0755	5.5619	43,421,030	
		No.2.	118	110	109	120	120					
		No.3.	117	107	118	109	109					
		No.4.	127	115	127	127	118					
		No.5.	128	115	126	128	119					
	Dry "uncoated" Bacterium coli after treatment with petroleum ether for 20 minutes	No.1.	140	148	146	136	134	146.96	2.1750	4.5684	5,440,459	12.53
		No.2.	147	151	140	140	153					
		No.3.	153	140	154	141	150					
		No.4.	151	148	154	161	150					
		No.5.	152	140	152	140	153					

TABLE 40 Cont'd.

Experiment		Sample Tube	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
III	Dry "uncoated" Bacterium coli	No.1.	72	61	70	69	62	69.4	1.8414	5.5619	25,314,344	
		No.2.	75	80	69	78	70					
		No.3.	70	61	60	69	60					
		No.4.	73	78	69	78	70					
		No.5.	74	64	65	75	63					
	Dry "uncoated" Bacterium coli after treatment with petroleum ether for 20 minutes	No.1.	90	88	88	79	90	82.64	1.9172	4.5684	3,059,332	12.09
		No.2.	70	83	80	83	81					
		No.3.	80	76	76	69	78					
		No.4.	94	82	86	85	87					
		No.5.	79	92	80	80	80					

Mean average survival :- $\frac{12.43 + 12.53 + 12.09}{3}$ = 12.35%

TABLE 41

THE SURVIVAL OF DRY "UNCOATED" SPORES OF BACILLUS SUBTILIS AFTER TREATMENT WITH PETROLEUM ETHER (B.Pt. 40° - 60°C) for 20 MINUTES.

Experiment		Sample Tube	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of original suspension	Percentage Survival
I	Dry "uncoated" spores of Bacillus subtilis	No.1.	61	60	70	60	65	58.12	1.7643	8.9238	48801,734,000	
		No.2.	56	64	57	58	52					
		No.3.	50	59	57	56	57					
		No.4.	57	60	57	50	58					
		No.5.	60	54	62	58	65					
	Dry "uncoated" spores of Bacillus subtilis after treatment with petroleum ether for 20 mins.	No.1.	60	64	68	60	60	59.64	1.7755	8.9238	50066,549,030	102.6
		No.2.	60	66	58	53	60					
		No.3.	60	55	58	50	56					
		No.4.	60	62	66	58	58					
		No.5.	61	54	60	59	65					

TABLE 41 Cont'd.

Experiment		Sample Tube	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of original suspension	Percentage Survival
II	Dry "uncoated" spores of Bacillus subtilis	No.1.	90	96	95	103	90	96.28	1.9836	8.5250	32252,729,000	
		No.2.	94	96	98	90	102					
		No.3.	99	98	104	92	99					
		No.4.	96	104	97	[illegible]	96					
		No.5.	92	98	90	100	98					
	Dry "uncoated" spores of Bacillus subtilis after treatment with petroleum ether for 20 minutes.	No.1.	96	101	97	90	94	94.36	1.9748	8.5250	31613,928,000	98.1
		No.2.	97	95	90	98	100					
		No.3.	92	88	98	99	88					
		No.4.	92	87	98	90	88					
		No.5.	98	88	98	99	98					

40 and 41 respectively. There was nil mortality after 20 minutes contact between petroleum ether and Bacterium coli in freeze dried peptone powder, while a similar period of contact between dry "uncoated" Bacterium coli and petroleum ether resulted in between 87.91 and 87.47 per cent mortality. This indicated that in the former case the organisms did not come into direct contact with the solvent due to their being embedded in and thus protected by the peptone particles.

No viable spores of Bacillus subtilis were killed by petroleum ether (b.pt. 40° - 60°C) after contact for 20 minutes. Bullock and Keepe (1951) obtained similar results when they submitted spores of Bacillus subtilis in spray-dried peptone powder to the action of petroleum ether. Thus, because of the high resistance of the spores to petroleum ether, it was not possible to detect any further protective effect of a "coating" of nutritive materials.

(b) The survival of dried "uncoated" Bacterium coli and spores of Bacillus subtilis during storage in oils.

Viable counts were performed on fifty-drop samples of light liquid paraffin infected with either dried "uncoated" Bacterium coli or spores of Bacillus subtilis, immediately after the preparation of the suspensions, and at intervals during storage. (Tables 42 and 43). The suspension was

TABLE 42

THE SURVIVAL OF DRY "UNCOATED" BACTERIUM COLI IN LIGHT LIQUID PARAFFIN.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
I	0 Hours	No.1.	60	52	56	59	60	59.8	1.7767	3.3964	148961	
		No.2.	56	58	63	67	54					
		No.3.	69	60	58	60	58					
		No.4.	61	68	60	61	62					
		No.5.	56	61	52	63	61					
	1½ Hours	No.1.	61	69	60	57	69	60.8	1.7839	2.1903	9430	6.33
		No.2.	73	65	62	74	60					
		No.3.	62	54	63	66	55					
		No.4.	64	54	53	65	53					
		No.5.	65	55	53	66	53					

TABLE 42 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival.
I	3 Hours	No.1.	33	34	28	36	28	34.4	1.5366	2.1903	5332	3.58
		No.2.	36	32	42	32	41					
	4½ Hours	No.1.	25	18	18	24	18	23.2	1.3655	2.1903	3596	2.41
		No.2.	28	21	30	21	29					
	6 Hours	No.1.	20	26	20			23.83	1.3772	1.8921	1858	1.25
		No.2.	28	29	20							
	24 Hours	No.1.	0	0	0			0.00	0.0000	1.8921	000	0.00
		No.2.	0	0	0							

TABLE 42 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	0 Hours	No.1.	225	207	202	220	200	213.00	2.3284	3.3964	530796	
		No.2.	210	214	227	231	212					
		No.3.	230	208	207	229	217					
		No.4.	200	218	198	217	220					
		No.5.	200	194	212	213	214					
	1½ Hours	No.1.	78	65	78	77	66	69.00	1.8388	3.3964	171948	32.39
		No.2.	60	72	60	61	73					
		No.3.	81	67	80	67	77					
		No.4.	61	70	72	62	62					
		No.5.	65	75	68	64	64					

TABLE 42 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	3 Hours	No.1.	140	124	127	140	136	131.00	2.1173	2.4928	40741	7.68
		No.2.	124	125	135	123	136					
	4½ Hours	No.1.	56	46	46	56	55	49.00	1.6902	2.4928	15239	2.87
		No.2.	45	41	50	51	42					
	6 Hours	No.1.	22	19	18	15	23	20.20	1.3054	2.1903	3131	0.59
		No.2.	25	17	16	24	23					
	24 Hours	No.1.	0	0	0			0.0	0.0000	1.8921	000	0.00
		No.2.	0	0	0							

TABLE 42 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
III	0 Hours	No.1.	77	72	81	80	74	77.08	1.8869	3.3964	192006	
		No.2.	76	82	87	84	79					
		No.3.	74	71	79	80	74					
		No.4.	70	76	69	79	77					
		No.5.	77	71	81	80	77					
	1½ Hours	No.1.	118	126	114	124	123	121.6	2.0849	2.1903	13848	9.81
		No.2.	120	118	112	121	121					
		No.3.	127	132	124	120	133					
		No.4.	124	117	113	123	114					
		No.5.	130	119	120	118	129					

TABLE 42 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil.	Percentage Survival.
III	3 Hours	No.1.	70	74	67	78	79	74.8	1.8739	2.1903	11594	6.04
		No.2.	84	71	84	70	71					
	4½ Hours	No.1.	59	64	72			68.16	1.8335	1.8921	5316	2.77
		No.2.	74	76	64							
	6 Hours	No.1.	24	19	17			20.5	1.3118	1.8921	1599	0.83
		No.2.	17	27	19							
	24 Hours	No.1.	0	0	0			0.00	0.00	1.8921	000	0.00
		No.2.	0	0	0							

TABLE 42 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
IV	0 Hours	No.1.	187	175	190	174	184	178.0	2.2504	3.3964	443398	
		No.2.	180	169	179	176	163					
		No.3.	190	174	186	186	173					
		No.4.	182	166	181	179	163					
		No.5.	180	184	182	169	180					
	1½ Hours	No.1.	90	80	76	77	88	78.8	1.8965	3.3964	196290	44.27
		No.2.	87	73	72	86	83					
		No.3.	81	80	69	79	68					
		No.4.	83	73	68	79	68					
		No.5.	91	80	76	79	84					

TABLE 42 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
IV	3 Hours	No.1.	114	118	119	107	121	116.4	2.0660	2.4928	36200	8.17
		No.2.	121	124	109	108	123					
	4½ Hours	No.1.	100	97	106	92	106	102.1	2.0090	2.1903	15825	3.57
		No.2.	107	109	96	110	98					
	6 Hours	No.1.	35	39	30			39.5	1.5966	1.8921	3081	0.69
		No.2.	47	49	37							
	24 Hours	No.1.	0	0	0			0.00	0.0000	1.8921	900	0.00
		No.2.	0	0	0							

TABLE 43

THE SURVIVAL OF DRY "UNCOATED" SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
I	0 Hours	No.1.	70	64	75	68	71	69.76	1.8436	6.4446	194,240,550	
		No.2.	72	80	75	71	67					
		No.3.	75	67	70	68	76					
		No.4.	69	61	68	68	69					
		No.5.	70	64	69	74	63					
	3 Hours	No.1.	81	70	70	70	71	70.72	1.8495	6.4446	196,913,590	101.3
		No.2.	76	68	68	62	68					
		No.3.	74	60	68	72	68					
		No.4.	81	72	70	69	70					
		No.5.	70	68	70	82	70					

TABLE 43 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
I	24 hours	No.1.	68	70	76	62	69	69.64	1.8428	6.4446	193,906,420	99.85
		No.2.	70	78	70	70	67					
		No.3.	72	66	60	68	74					
		No.4.	72	77	66	66	72					
		No.5.	70	76	69	62	70					
	72 hours	No.1.	129	142	131	141	134	138.0	2.1399	6.1440	192,234,000	99.02
		No.2.	136	145	134	130	136					
		No.3.	140	149	138	150	135					
	96 hours	No.1.	109	104	111	101	107	104.8	2.0203	6.1440	146,024,040	75.2
		No.2.	118	103	104	106	107					
		No.3.	102	98	110	97	102					

TABLE 43 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
I	8 days	No.1.	100	112	109	108	118	108.27	2.0342	6.1440	153,235,640	78.9
		No.2.	114	100	100	103	113					
		No.3.	110	114	107	116	100					
	15 days	No.1.	114	102	108	106	100	102.33	2.0098	6.1440	142,698,330	73.5
		No.2.	98	107	104	94	100					
		No.3.	100	100	109	100	113					
	30 days	No.1.	54	64	60	61	60	61.0	1.7782	6.1440	83,008,900	42.7
		No.2.	63	70	61	62	60					
		No.3.	60	65	61	55	59					
	60 days	No.1.	38	36	42	34	37	36.9	1.5670	5.8465	25,919,697	13.4
		No.2.	36	39	33	39	35					
	100 days	No.1.	99	86	90	95	92	93.8	1.9722	5.1506	13,267,822	6.8
		No.2.	97	102	92	93	92					

TABLE 43 Cont'd

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	0 Hours	No.1.	133	125	132	128	136	129.2	2.1113	6.4442	359,315,148	
		No.2.	130	132	122	132	119					
		No.3.	140	126	126	141	128					
		No.4.	125	114	126	127	128					
		No.5.	130	125	128	140	137					
	3 Hours	No.1.	128	119	130	129	119	128.8	2.1100	6.4442	358,202,717	99.69
		No.2.	140	142	132	130	134					
		No.3.	120	112	124	120	119					
		No.4.	120	134	133	134	126					
		No.5.	140	138	128	129	140					
	24 hours	No.1.	132	120	128	123	129	130.1	2.1142	6.4442	361,813,177	100.69
		No.2.	138	128	130	140	133					

TABLE 43 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	72 Hours	No.1.	127	117	128	121	127	128.0	2.1072	6.4442	355,968,000	99.07
		No.2.	136	125	130	139	130					
	96 Hours	No.1.	100	113	106	101	100	106.3	2.0265	6.4442	295,628,485	82.29
		No.2.	114	110	109	108	102					
	8 days	No.1.	102	109	97	94	98	102.5	2.0107	6.4442	285,060,392	79.33
		No.2.	109	112	108	100	96					
	15 days	No.1.	99	100	114	110	100	102.1	2.0090	6.4442	283,947,961	79.02
		No.2.	106	104	104	93	91					
	30 days	No.1.	115	116	105	101	113	107.9	2.0331	6.1433	150,163,566	41.79
		No.2.	104	101	107	114	103					

TABLE 43 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	60 days	No.1.	169	149	170	167	149	166.2	2.2206	5.4487	46,568,076	12.96
		No.2.	180	178	160	160	180					
	100 days	No.1	94	91	81	81	94	91.9	1.9633	5.4487	25,749,736	7.16
		No.2.	102	89	100	88	99					

constantly rotated about horizontal axis during the period of storage. When calculating the survivors over the different periods allowance was made for mortality in petroleum ether.

An Analysis of Variance of the viable counts performed immediately after the preparation of the oily suspension of dried "uncoated" Bacterium coli suggested that equal volumes of the infected oil (0.52 millilitre samples) gave rise to approximately equal number of colonies in the roll tubes after incubation. Thus it was deduced at this stage that the organisms were uniformly dispersed throughout the oil. A similar conclusion was recorded with regard to the suspensions of dried "uncoated" spores of Bacillus subtilis in the oil (Tables 44 and 45).

The observed viability of dried "uncoated" Bacterium coli in light liquid paraffin varied substantially from experiment to experiment. The cause of the between experiment variation was not immediately apparent but later experiments suggested a possible explanation (page 304).

The mortality of both Bacterium coli and spores of Bacillus subtilis in the oil was considerably greater than that observed when they were stored in the dried state (Tables 36 and 39 and Figs. III and VI). Further, the mortality of dried "uncoated" organisms in light liquid

paraffin was considerably greater than that of Bacterium coli, which had been freeze-dried from peptone solution, in the same oil. In none of the experiments performed were viable organisms found after 24 hours storage in light liquid paraffin. It can, therefore, be concluded that light liquid paraffin has a marked bactericidal action on dry Bacterium coli and that the severity of the lethal action is reduced when the organisms are coated with nutrient materials.

TABLE 44

ANALYSIS OF VARIANCE OF QUINTUPLICATE ROLL-TUBING OF FIVE SAMPLES FROM THE SAME LIGHT LIQUID PARAFFIN CONTAINING DRY "UNCOATED" BACTERIUM COLI.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
I	(a) Between samples	77	4	19.25	1.049	>0.2
	(b) Within samples	367	20	18.35		
	Total	444	24			
II	(a) Between samples	561	4	140.25	1.319	>0.2
	(b) Within samples	2127	20	106.35		
	Total	2688	24			

TABLE 45

ANALYSIS OF VARIANCE OF QUINTUPLICATE ROLL-TUBING OF FIVE SAMPLES FROM THE SAME LIGHT LIQUID PARAFFIN CONTAINING DRY "UNCOATED" SPORES OF BACILLUS SUBTILIS.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
I	(a) Between samples	117	4	29.25	1.242	>0.2
	(b) Within samples	471	20	23.55		
	Total	588	24			
II	(a) Between samples	256	4	64.0	1.721	0.1-0.2
	(b) Within samples	744	20	37.2		
	Total ...	1000	24			

The inescapable conclusion that light liquid paraffin is bactericidal to vegetative cells is at variance with that reached by Bullock and Keepe (1951). The latter authors (1951) cast doubt on the opinion of many pharmacists "that oils are self-sterilising at least as regards vegetative bacteria". They stated that "vegetative cells die off rapidly in oily media, but as they do the same apparently

in the dry powders, their destruction is not a function of the oil. The results show quite clearly that oils are not reasonably self-sterilising. Large numbers of even vegetative cells will survive 6 months storage in arachis oil".

The conclusion reached by Bullock and Keepe (1951) that oils are not bactericidal to vegetative cells may be attributed to the fact that the organisms employed in their experiments were coated with the coating of peptone. Such a coating inhibits the passage of an oil towards the bacterial surface and thereby behaves as an effective barrier against the oil. It is probable that the organisms employed by the above-mentioned authors were at no time in direct contact with the oil and thus were never subjected to its bactericidal action. The times quoted by Bullock and Keepe (1951) for survival of vegetative cells in oils were in fact, survival times for cells which were heavily coated with nutrient materials and thus protected from the direct action of oils.

Spores of Bacillus subtilis died much more slowly in light liquid paraffin than did Bacterium coli (Tables 43 and Fig. VI). After 8 days storage between 78.9 and 79.3 per cent of the spores were viable, while after 100 days only between 6.8 and 7.2 per cent were still viable. When a

sample of the same batch of spores was stored in a sealed tube in the dried condition there were 100 per cent survivors after 100 days (Table 39 and Fig. VI). Thus light liquid paraffin is mildly bactericidal to spores. This mild bactericidal action was not observed by Bullock and Keepe (1951) who reported negligible mortality when Bacillus subtilis spores were stored in arachis oil for 12 months. The smaller mortality observed by these authors can again probably be attributed to the fact that their spray-dried spores were coated with some protective material.

An examination of the survival of dry "uncoated" Bacterium coli in arachis oil and of spores of Bacillus subtilis in both arachis oil and mixture of liquid paraffins further confirmed the bactericidal action of oils (Tables 46, 47 and 48). No dried "uncoated" Bacterium coli survived longer than 24 hours in arachis oil. This indicated that arachis oil was as bactericidal to this organism as was light liquid paraffin.

Table 47 and Fig. VI show that arachis oil was much more bactericidal to the spores of Bacillus subtilis than either light liquid paraffin or the mixture of paraffins.

TABLE 46

THE SURVIVAL OF DRY "UNCOATED" BACTERIUM COLI IN ARACHIS OIL.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil	Percentage Survival
I	0 Hour	No.1.	76	70	81	82	71	72.12	1.8580	3.3209	151,019	
		No.2.	73	85	86	83	76					
		No.3.	78	70	79	78	69					
		No.4.	70	76	66	77	76					
		No.5.	77	69	80	80	75					
	$1\frac{1}{2}$ Hours	No.1.	140	130	128	141	131	136.70	2.1358	2.1644	19958	13.21
		No.2.	147	135	134	136	145					
	3 Hours	No.1.	67	64	64	75	75	72.00	1.8573	2.1644	10512	6.96
		No.2.	79	79	69	71	77					

TABLE 46 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil	Percentage Survival
I	4½ Hours	No.1.	140	130	145	133	133	138.50	2.1415	1.5441	4847	3.21
		No.2.	147	134	146	143	134					
	6 Hours	No.1.	147	157	160			155.33	2.1917	0.9395	1351	0.90
		No.2.	154	150	164							
	24 Hours	No.1.	0	0	0			0.00	0.0000	0.9395	0	0.00
		No.2.	0	0	0							

TABLE 46 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml of oil	Percentage Survival
II	0 Hour	No.1.	79	70	83	80	71	78.12	1.8928	3.3209	163,583	
		No.2.	76	87	87	81	77					
		No.3.	81	71	82	79	70					
		No.4.	74	81	70	79	82					
		No.5.	79	70	82	82	73					
	$1\frac{1}{2}$ Hours	No.1.	137	129	130	143	129	135.30	2.1313	2.4440	37613	23.00
		No.2.	141	132	131	139	142					
	3 Hours	No.1.	89	94	90	99	87	94.40	1.9750	2.1644	13782	8.43
		No.2.	100	102	91	92	100					

TABLE 46 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml of oil	Percentage Survival
II	$4\frac{1}{2}$ Hours	No.1.	132	140	137	141	139	141.70	2.1513	1.5441	4959	3.03
		No.2.	140	139	149	150	150					
	6 Hours	No.1.	151	149	164			156.83	2.1952	0.9395	1364	0.84
		No.2.	160	167	154							
	24 Hours	No.1.	0	0	0			0.00	0.0000	0.9395	0	0.00
		No.2.	0	0	0							

TABLE 47

THE SURVIVAL OF DRY "UNCOATED" SPORES OF BACILLUS SUBTILIS IN ARACHIS OIL.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage survival
I	0 Hours	No.1.	87	89	100	86	101	88.24	1.9457	6.4006	221,928,894	
		No.2.	88	75	87	84	78					
		No.3.	91	86	90	89	100					
		No.4.	95	82	84	94	83					
		No.5.	91	89	89	78	90					
	3 Hours	No.1.	87	75	87	86	77	88.56	1.9472	6.4006	222,733,713	100.36
		No.2.	91	88	89	89	100					
		No.3.	85	97	100	85	99					
		No.4.	95	83	83	95	83					
		No.5.	90	91	90	79	90					

TABLE 47 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
I	24 Hours	No.1.	80	80	92	81	89	85.9	1.9340	6.4006	216,043,654	97.35
		No.2.	92	82	90	80	93					
	96 Hours	No.1.	70	70	73	82	70	75.0	1.8751	6.4006	188,629,500	85.00
		No.2.	78	71	84	70	82					
	8 days	No.1.	62	55	50	50	51	54.9	1.7396	6.4006	138,076,794	62.21
		No.2.	61	62	50	58	50					
	15 days	No.1.	141	130	129	134	143	132.0	2.1206	5.8023	83,736,180	37.73
		No.2.	133	134	122	120	134					

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
I	30 days	No.1.	184	190	174	187	172	184.4	2.2657	5.4038	46,725,484	21.06
		No.2.	200	181	182	180	194					
	60 days	No.1.	160	144	160	159	150	148.4	2.1715	5.4038	37,603,372	16.94
		No.2.	146	135	149	135	146					
	100 days	No.1.	48	40	50	42	43	43.9	1.6425	5.4038	11,124,260	5.01
		No.2.	45	46	39	46	40					

TABLE 47 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	0 Hours	No.1.	147	130	134	136	138	136.76	2.1358	6.0997	172,122,580	
		No.2.	149	133	148	135	149					
		No.3.	138	136	125	137	125					
		No.4.	150	149	136	134	136					
		No.5.	139	139	126	124	126					
	3 Hours	No.1.	149	131	144	130	134	136.76	2.1358	6.0997	172,122,580	100.00
		No.2.	138	124	137	138	125					
		No.3.	151	149	134	134	135					
		No.4.	149	150	131	134	147					
		No.5.	138	138	125	127	127					
	24 Hours	No.1.	133	120	124	129	131	131.9	2.1202	6.0997	166,005,910	96.45
		No.2.	144	130	132	145	131					

TABLE 47 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	96 Hours	No.1.	117	116	109	116	104	116.9	2.0679	6.0997	147,127,300	85.48
		No.2.	127	116	118	130	116					
	8 days	No.1.	66	80	79	80	77	73.7	1.8675	6.0997	92,756,903	53.89
		No.2.	80	78	66	66	65					
	15 days	No.1.	191	208	190	188	204	199.50	2.3000	5.4038	50,551,704	29.38
		No.2.	204	199	195	214	202					
	30 days	No.1.	150	134	136	151	146	150.00	2.1761	5.4038	38,008,800	22.08
		No.2.	160	148	149	167	159					

TABLE 47 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	60 days	No.1.	108	98	95	110	98	105.80	2.0245	5.4038	26,808,873	15.57
		No.2.	118	103	102	119	104					
	100 days	No.1.	39	36	43	37	40	40.1	1.6031	5.4038	10,131,340	5.90
		No.2.	45	38	39	45	39					

TABLE 48

THE SURVIVAL OF DRY "UNCOATED" SPORES OF BACILLUS SUBTILIS IN A MIXTURE OF LIQUID PARAFFINS.

(50 PARTS OF LIGHT LIQUID PARAFFIN AND 50 PARTS OF LIQUID PARAFFIN).

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil.	Percentage Survival.
I	0 Hours	No.1.	203	204	200	210	216	204.72	2.3111	5.7592	117,596,286	
		No.2.	210	195	208	190	193					
		No.3.	204	196	210	201	193					
		No.4.	212	218	216	202	204					
		No.5.	200	199	214	216	204					
	3 Hours	No.1.	210	201	202	203	217	205.92	2.3137	5.7592	118,285,596	100.59
		No.2.	214	196	210	194	192					
		No.3.	210	200	216	201	198					
		No.4.	214	220	215	201	202					
		No.5.	200	198	215	215	204					

TABLE 48 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
I	24 hours	No.1.	100	197	106	102	109	104.70	2.0199	6.0565	119,321,669	101.47
		No.2.	102	102	104	109	116					
	96 Hours	No.1.	99	104	97	105	96	102.7	2.0115	6.0565	117,042,363	99.53
		No.2.	101	99	110	115	101					
	8 days	No.1.	98	104	96	106	97	102.5	2.0107	6.0565	116,814,432	99.34
		No.2.	102	99	109	114	100					
	15 days	No.1.	90	93	100	102	91	93.4	1.9703	6.0565	106,443,590	90.52
		No.2.	90	96	90	86	96					

TABLE 48 Cont'd

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
I	30 days	No.1.	82	80	80	88	91	81.9	1.9133	6.0565	93,337,580	79.37
		No.2.	80	79	89	80	80					
	60 days	No.1.	128	140	126	129	127	132.0	2.1206	5.7592	75,824,100	64.48
		No.2.	132	140	138	126	134					
	100 days	No.1.	94	96	102	99	106	97.9	1.9908	5.7592	56,236,207	47.82
		No.2.	101	99	100	92	90					

TABLE 48 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil.	Percentage Survival
II	0 Hour	No.1.	142	142	131	128	133	132.64	2.1225	5.7592	76,191,732	
		No.2.	130	129	132	117	118					
		No.3.	136	130	130	143	130					
		No.4.	130	142	130	135	129					
		No.5.	133	133	135	135	123					
	3 Hours	No.1.	145	132	132	140	134	132.76	2.1229	5.7592	76,260,663	100.09
		No.2.	130	128	134	119	120					
		No.3.	139	130	129	141	130					
		No.4.	130	142	129	136	139					
		No.5.	130	133	136	136	125					

TABLE 48 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	24 Hours	No.1.	60	70	69	66	60	66.3	1.8215	6.0565	75,559,060	99.17
		No.2.	67	70	68	71	62					
	96 Hours	No.1.	60	71	68	65	60	66.1	1.8202	6.0565	75,331,129	98.87
		No.2.	67	72	68	71	59					
	8 days	No.1.	72	60	70	60	64	66.0	1.8195	6.0565	75,217,164	98.72
		No.2.	63	60	72	69	70					
	15 days	No.1.	60	69	58	69	59	61.8	1.7910	6.0565	70,430,617	92.44
		No.2.	61	58	58	67	59					

TABLE 48 Cont'd.

Experiment	Duration of Storage	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of oil	Percentage Survival
II	30 days	No.1.	89	96	100	87	85	94.8	1.9768	5.7592	54,455,490	71.47
		No.2.	102	96	101	103	89					
	60 days	No.1.	78	80	70	82	70	77.8	1.8910	5.7592	44,690,265	58.66
		No.2.	80	67	81	80	70					
	100 days	No.1.	60	70	58	59	66	64.5	1.8096	5.7592	37,050,412	48.63
		No.2.	70	71	61	71	59					

(D) THE MIGRATION OF DRY "UNCOATED" SPORES OF BACILLUS SUBTILIS FROM LIGHT LIQUID PARAFFIN TO RINGER'S SOLUTION.

When oil containing Bacterium coli embedded in peptone was floated over sterile Ringer's solution, all the organisms initially present in the oil passed into the Ringer's solution within 30 minutes (Page 99). It was deduced from this observation that the minimal size of the particles in the suspension was about 810 μ, which suggested that the organisms were present in the oil either as aggregates or embedded in large particles of peptone. The dimensions of individual "uncoated" Bacterium coli or spores of Bacillus subtilis would not exceed approximately 2 μ. The rate of sedimentation through the oil of organisms having such dimensions was expected to be much less than that observed for peptone embedded organisms. Dry "uncoated" Bacterium coli died rapidly in light liquid paraffin and were, therefore, considered unsatisfactory for sedimentation studies. Because dry "uncoated" spores of Bacillus subtilis suffered less than 1.0 per cent mortality in light liquid paraffin within 72 hours, they were selected for infecting the oil for sedimentation studies (Table 43 and Fig.VI).

The design of the experiments to study the rate of

migration of dry "uncoated" spores of Bacillus subtilis through an oil to an underlying aqueous phase was similar to that described on page 93 . As no prior information was available regarding the time required for complete migration of the spores from the oil to the aqueous phase, it was necessary also to know the mortality rate of spores in the Ringer's solution. Table 49 records nil mortality of the spores during 7 days storage in the Ringer's solution.

I. Determination of Reproducibility of the Viable Counts of Dry "Uncoated" Spores of Bacillus subtilis Suspended in Light Liquid Paraffin in Different Separators.

0.52 millilitre samples of light liquid paraffin infected with Bacterium coli in peptone contained equal numbers of viable organisms, provided the oil was rotated continuously so as to prevent sedimentation. (Table 17). Equal volumes of the same oil taken after 15 minutes stationary floatation over sterile Ringer's solution no longer contained equal numbers of viable organisms, because the rate of sedimentation was not uniform from separator to separator (Tables 31 and 32). It was, thus, not possible to regard the count of the sample taken from any one separator as indicative of the count of a similar sample from any other separator.

TABLE 49

THE SURVIVAL OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
0 Hour	96	102	99	108	96	100.2	2.0008	5.6373	43,472,772	
3 Hours	109	101	96	100	100	101.2	2.0051	5.6373	43,906,632	101.0
6 Hours	106	103	100	96	102	101.4	2.0060	5.6373	43,993,404	101.2
24 Hours	108	96	96	105	101	101.2	2.0051	5.6373	43,906,632	101.0
72 Hours	107	103	98	95	96	99.8	1.9991	5.6373	43,299,228	99.6
168 Hours	94	99	92	104	103	98.6	1.9939	5.6373	42,778,596	98.4

In order to determine whether or not dry "uncoated" spores of Bacillus subtilis sedimented at a more uniform rate than did the organisms embedded in peptone, oil containing dry "uncoated" spores of Bacillus subtilis was floated over sterile Ringer's solution in 10 different separators. It was anticipated that sedimentation would be much slower than that observed when using peptone-coated organisms. Viable counts, were, therefore, performed on samples of oil taken from each of five separators after the two-phases had been in contact for 1½ hours and from each of the remaining five separators after 3 hours. Viable counts were also performed on the Ringer's solutions removed from the same separators when the oil samples were taken (Table 50).

Prior to floatation over the Ringer's solution equal volumes of the infected oil, which was contained in a tube rotated about a horizontal axis, contained equal numbers of spores. (Tables 43 and 45). The viable counts on samples of oil removed from 5 separators after 1½ hours contact with the Ringer's solution, were very much more uniform than those obtained from oil infected with peptone-coated Bacterium coli which had floated on Ringer's solution for 15 minutes. An Analysis of Variance of the counts recorded in Table 50 indicated that the variation in the counts on the oil samples removed from 5 separators was not significantly greater than the "within separator" variation (Table 51). An exactly similar condition

TABLE 50

VARIATION OF COUNTS OF SPORES OF BACILLUS SUBTILIS IN OIL AND RINGER'S SOLUTION FROM DIFFERENT SEPARATORS.

	Duration of Contact	Counts from different separators.				
		1	2	3	4	5
Oil	1½ Hours	80 70 81 80 89	92 82 92 82 84	82 72 83 73 81	90 81 90 79 80	82 86 72 74 84
	3 Hours	69 61 71 62 65	74 76 65 66 76	79 70 70 79 70	67 60 72 72 61	70 72 63 72 63
Ringer's Solution	1½ Hours	179 187 175 177 186	162 175 171 174 179	170 181 183 170 172	168 180 167 177 168	165 175 164 174 180
	3 Hours	102 116 115 103 102	112 124 110 124 110	107 117 103 117 104	110 109 124 121 120	110 107 120 118 118

TABLE 51

ANALYSIS OF VARIANCE OF COUNTS OBTAINED FROM OIL AND RINGER'S SOLUTION FROM DIFFERENT SEPARATORS.

	After Sedimentation time	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
Oil	1½ Hours	(a) Between separators	235	4	58.75	1.686	0.1-0.2
		(b) Within separators	697	20	34.85		
		Total	932	24			
	3 Hours	(a) Between separators	231	4	57.75	2.260	0.05 - 0.1
		(b) Within separators	511	20	25.55		
		Total	742	24			
Ringer's Solution	1½ Hours	(a) Between separators	300	4	75.0	1.938	0.1-0.2
		(b) Within separators	774	20	38.7		
		Total	1074	24			
	3 Hours	(a) Between separators	334	4	83.5	1.317	>0.2
		(b) Within separators	1268	20	63.4		
		Total	1602	24			

was obtained after the oil had been in contact with the Ringer's solution for 3 hours (Table 51).

It can, therefore, be concluded that the rate of sedimentation through light liquid paraffin of dry "uncoated" spores of Bacillus subtilis is uniform from separator to separator whereas that of peptone coated organisms was far from uniform. Confirmation of the much more uniform rate of sedimentation of dry "uncoated" spores is provided by the viable counts on the samples of Ringer's solution (c.f. Tables 31 and 33). Thus, provided "uncoated" organisms are used to infect the oil, a viable count performed on a sample of oil or of Ringer's solution removed from any one separator of a series can be regarded as representing the viable count of similar samples removed from any of the remaining separators.

II. The Migration of Dry "Uncoated" Spores of Bacillus subtilis from Light Liquid Paraffin to Ringer's Solution.

Light liquid paraffin containing a known number of spores of Bacillus subtilis per unit volume was floated over sterile Ringer's solution in a series of separators. After allowing the two phases to remain in contact for specified intervals of time, 0.52 millilitre samples of oil were carefully taken from a separator at each time interval at depths of 1, 2 and 3 centimetres below the surface, and the Ringer's solution

was also collected from the same separator at each time. The number of viable organisms remaining at each of the 3 levels, and hence the number of viable organisms which had sedimented through each of the levels, was calculated. The mean number of organisms which had sedimented per unit volume of oil in the separator and the number of viable organisms which passed through the oil into the Ringer's solution during each time interval was also calculated (Tables 52-56).

(a) Sedimentation of dry "uncoated" spores of Bacillus subtilis through light liquid paraffin.

In an aqueous suspension the bacterial cell is in osmotic equilibrium with the water and hence the density of the cell can be regarded as approximately 1.0. Assuming the density of the spore of Bacillus subtilis to be 1.0 and its diameter to be 1.0 micron (Topley and Wilson - 1948 and Burgey - 1948), it was estimated from the Stoke's law equation that the time required for it to sediment through a distance of 1 centimetre in light liquid paraffin would be approximately 8.66×10^4 hours. The minimal size of the particles that would sediment through the above three distances during each time interval was calculated to range from 416 to 34 microns (Table 56).

The theoretical minimal size of particles that would

TABLE 52

SEDIMENTATION OF SPORES OF BACILLUS SUBTILIS THROUGH 1 CENTIMETRE OF LIGHT LIQUID PARAFFIN.

Initial number of organisms in oil :- 98,016,000/ml.

Time of sedimentation	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil	Number of organisms sedimenting per ml. of oil	Percentage of initial number of organisms sedimenting in oil.
1½ Hours	89	102	92	102	102	97.4	1.9886	5.9243	81,806,747	16,210,166	16.54
3 Hours	73	85	86	74	88	81.2	1.9096	5.9243	68,200,286	29,816,627	30.42
6 Hours	63	75	76	65	67	69.2	1.8401	5.9243	58,121,426	39,895,487	40.70
24 Hours	169	184	183	171	173	176.0	2.2455	4.8390	12,152,096	85,864,817	87.60
72 Hours	95	109	96	109	105	102.8	2.0119	4.5383	3,551,020	94,465,893	96.38

TABLE 53

SEDIMENTATION OF SPORES OF BACILLUS SUBTILIS THROUGH .2 CENTIMETRES OF LIGHT LIQUID PARAFFIN.

Initial number of organisms in oil :- 98,016,000/ml.

Time of sedimentation	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil	Number of organisms sedimenting per ml. of oil	% of initial number of organisms sedimenting in oil
1½ Hours	96	109	100	102	98	101.0	2.0043	5.9243	84,830,405	13,186,508	13.45
3 Hours	81	92	84	93	94	88.8	1.9484	5.9243	74,583,564	23,433,349	23.91
6 Hours	63	75	76	65	78	71.4	1.8537	5.9243	59,969,217	38,047,696	38.82
24 Hours	168	186	185	172	186	179.4	2.2539	4.8390	12,386,852	85,630,061	87.36
72 Hours	99	109	108	98	109	104.6	2.0195	4.5383	3,613,197	94,403,716	96.31

TABLE 54

SEDIMENTATION OF SPORES OF BACILLUS SUBTILIS THROUGH 3 CENTIMETRES OF LIGHT LIQUID PARAFFIN.

Initial number of organisms in oil :- 98,013,000/ml.

Time of sedimentation	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil	Number of organisms sedimenting per ml. of oil	Percentage of initial number of organisms sedimenting in oil
1½ Hours	96	107	98	106	108	103.0	2.0128	5.9243	86,510,215	11,506,698	11.74
3 Hours	92	104	94	102	98	98.0	1.9912	5.9243	82,310,690	15,706,223	16.02
6 Hours	70	81	69	80	82	76.4	1.8831	5.9243	64,168,742	33,848,171	34.53
24 Hours	170	187	172	189	187	181.0	2.2577	4.8390	12,497,326	85,519,587	87.30
72 Hours	102	114	114	100	109	107.8	2.0327	4.5383	3,723,735	94,293,178	96.20

TABLE 55

MIGRATION OF SPORES OF BACILLUS SUBTILIS FROM LIGHT LIQUID PARAFFIN TO RINGER'S SOLUTION.

Total number of organisms in oil :- 1470,253,695.

Time of contact between two phases.	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms in Ringer's solution	Percentage of initial number of organisms (in the oil) migrating to Ringer's solution.
1½ Hours	270	249	267	254	253	258.6	2.4126	5.1568	37,134,184	2.52
3 Hours	164	177	165	178	176	172.0	2.2355	5.6700	80,472,952	5.47
6 Hours	232	250	252	233	253	242.0	2.3838	5.6700	113,223,572	7.70
24 Hours	84	97	97	84	94	91.8	1.9628	6.2422	160,374,875	10.91
72 Hours	133	148	146	133	130	138.0	2.1399	6.2422	241,086,414	16.40

TABLE 56

SEDIMENTATION OF SPORES OF BACILLUS SUBTILIS THROUGH LIGHT LIQUID PARAFFIN AND THEIR MIGRATION INTO RINGER'S SOLUTION.

Sedimentation Time	Organisms sedimenting through						Mean % of initial number of organisms sedimenting through oil	% of initial number of organisms migrating into Ringer's solution
	1 Centimetre		2 Centimetres		3 Centimetres			
	Theoretical minimal size of aggregates in microns	% of initial number of organisms	Theoretical minimal size of aggregates in microns	% of initial number of organisms	Theoretical minimal size of aggregates in microns	% of initial number of organisms		
1½ Hours	240	16.54	340	13.45	416	11.74	13.91	2.52
3 Hours	170	30.42	240	23.91	294	16.02	23.12	5.47
6 Hours	120	40.70	170	38.82	208	34.53	38.02	7.70
24 Hours	60	87.60	85	87.36	104	87.30	87.42	10.91
72 Hours	34	96.38	49	96.31	60	96.20	96.30	16.40

sediment through 1 centimetre of light liquid paraffin during 1½ and 3 hours is approximately 240 and 170 microns respectively. The number of organisms which actually sedimented this distance in light liquid paraffin during 1½ and 3 hours was about 16 and 30 millions respectively. This suggested that about 16 million organisms were present in the oil in the form of aggregates having dimensions greater than 240 microns while 30 million organisms were in the oil as aggregates having dimensions greater than 170 microns. These organisms could not be present as individual discreet cells, as the estimated time required for such discreet cells to sediment through 1 centimetre of light liquid paraffin is approximately 8.66×10^4 hours. It can be, thus, deduced from the sedimentation rates recorded in Table 56 that the organisms were present in the oil in the form of aggregates, the minimal dimensions of which ranged from about 34 microns to about 400 microns.

Bacterium coli in peptone freeze-dried powder were present in the form of particles with a minimal size of 810 microns. In the present experiment only about 12 per cent of the organisms were in aggregates of greater than 400 microns. The minimal size of aggregates was less than 34 microns and about 3 per cent of the organisms were in such small aggregates. Thus, the aggregates in the case of dry "uncoated" organisms were much smaller than those of organisms in peptone.

(b) Adhesion of spores of Bacillus subtilis during sedimentation in light liquid paraffin.

Adam (1949) stated that the sedimentation volume of particles sedimenting freely in a liquid depends on the density, size and shape of the solid particles; it is also found to depend markedly on the adhesion between the particles themselves. If the mutual adhesion between the particles is high, the particles tend to pack together into a volume very much greater than that occupied by the same particles when there is less adhesion between them. This increase in the volume of the particles would, therefore, increase the sedimentation volume per unit time.

The theoretical minimal size of particles which would sediment through distances of 1 and 2 centimetres of light liquid paraffin during 1½ and 3 hours respectively is the same, namely 240 microns (Table 56). The number of organisms in such particles would also be the same. In the experiment described, the number of organisms sedimenting through 1 centimetre of the oil during 1½ hours was about 16 millions, while the number of organisms sedimenting through 2 centimetres of the oil during 3 hours was about 23 millions. The observation that a greater number of

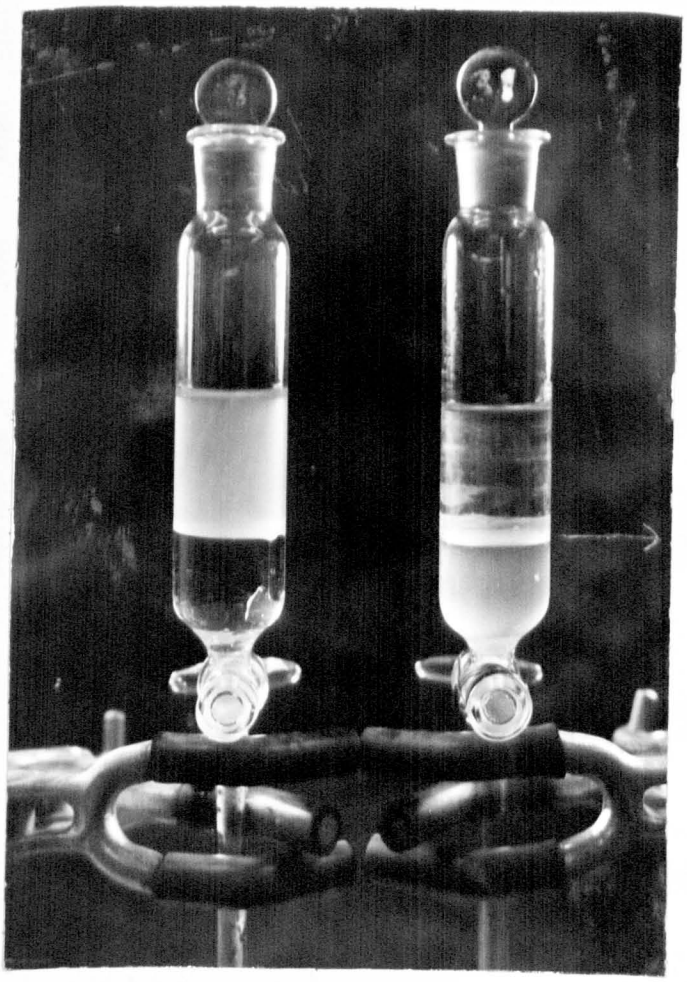
organisms sedimented through 2 centimetres during 3 hours than through 1 centimetre during 1½ hours, must mean that some of the aggregates which were sedimenting at 1½ hours adhered together between 1½ and 3 hours to produce larger aggregates. The larger aggregates so formed would sediment more rapidly and would result in an increased sedimentation volume resulting in an increase in the number of organisms sedimenting. Adhesion must have been similarly responsible for the greater number of organisms which sedimented through 2 centimetres of oil during 6 hours than sedimented through 1 centimetre during 3 hours, and so on.

(c) (i) The migration of spores of Bacillus subtilis from light liquid paraffin to Ringer's solution.

Initially, when oily and aqueous phases were brought together, the infected oily layer was visually turbid and the lower Ringer's solution was clear. After the two phases had remained in contact for 72 hours, the upper oily layer was clear and the Ringer's solution below it was turbid. During this period most of the organisms had sedimented through the oil and some of them had passed into the Ringer's solution (Table 55-56). At no time during the experiment did all the organisms which had sedimented through the oil appear in the Ringer's

solution. After 72 hours sedimentation only about 3 per cent of the initial number of organisms remained in the oil, and yet only about 16 per cent of the initial number had migrated into the Ringer's solution. The mortality of the organisms in the oil during 72 hours was less than 1.0 per cent and thus the loss of viable organisms from the suspension due to death was insignificant compared to the loss of viable organisms due to sedimentation. Thus, the majority of the organisms which sedimented through the oil must have been adsorbed at the interface which became distinctly white and opalescent (Plate I).

Mudd and Mudd (1924) observed that when oils were allowed to come into contact with an aqueous drop of bacterial suspension on a microscope slide, the organisms in the aqueous phase tended to concentrate at the interface. In the experiment described above, the organisms were under the influence of free gravitational force and therefore must have complied with the conditions enunciated by Adam (1949) for adsorption of solid particles at an interface. Adam (1949) stated that when, in a system containing solid particles distributed between two liquids, neither of the solid tensions (i.e., solid tension between the solid and either of the two liquids) exceeds the sum of the other solid tension (between the solid and the remaining liquid) and the interfacial tension (between the two



White opalescent interface containing adsorbed organisms.

Plate I. ADSORPTION OF ORGANISMS AT THE INTERFACE BETWEEN OIL AND RINGER'S SOLUTION.

Separator on the left :- Immediately after floatation of infected oil.

Separator on the right :-After sedimentation for 72 hours.

liquids), the solid is adsorbed at the interface. In the migration experiment described above, the organisms were in the oily phase and were sedimenting through the oil under gravitational influence and the majority of them, on reaching the interface, were adsorbed there. If adsorption at the interface is due to the fulfilment of the conditions enunciated by Adam, it should be possible to increase the number of organisms passing through the interface into the aqueous phase by reducing the interfacial tension. An attempt to achieve this is described below.

(ii) The determination of interfacial tension between oil and aqueous phase.

The interfacial tension between light liquid paraffin and different concentrations of Tween 80 in Ringer's solution was estimated by Du Nuoy's method and is plotted in Fig.VII. There was a marked decrease of interfacial tension as the concentration of Tween 80 in Ringer's solution was increased up to 0.8 per cent, in excess of which concentration there was no further reduction in the interfacial tension.

(iii) The survival of spores of Bacillus subtilis in Ringer's solution containing Tween 80.

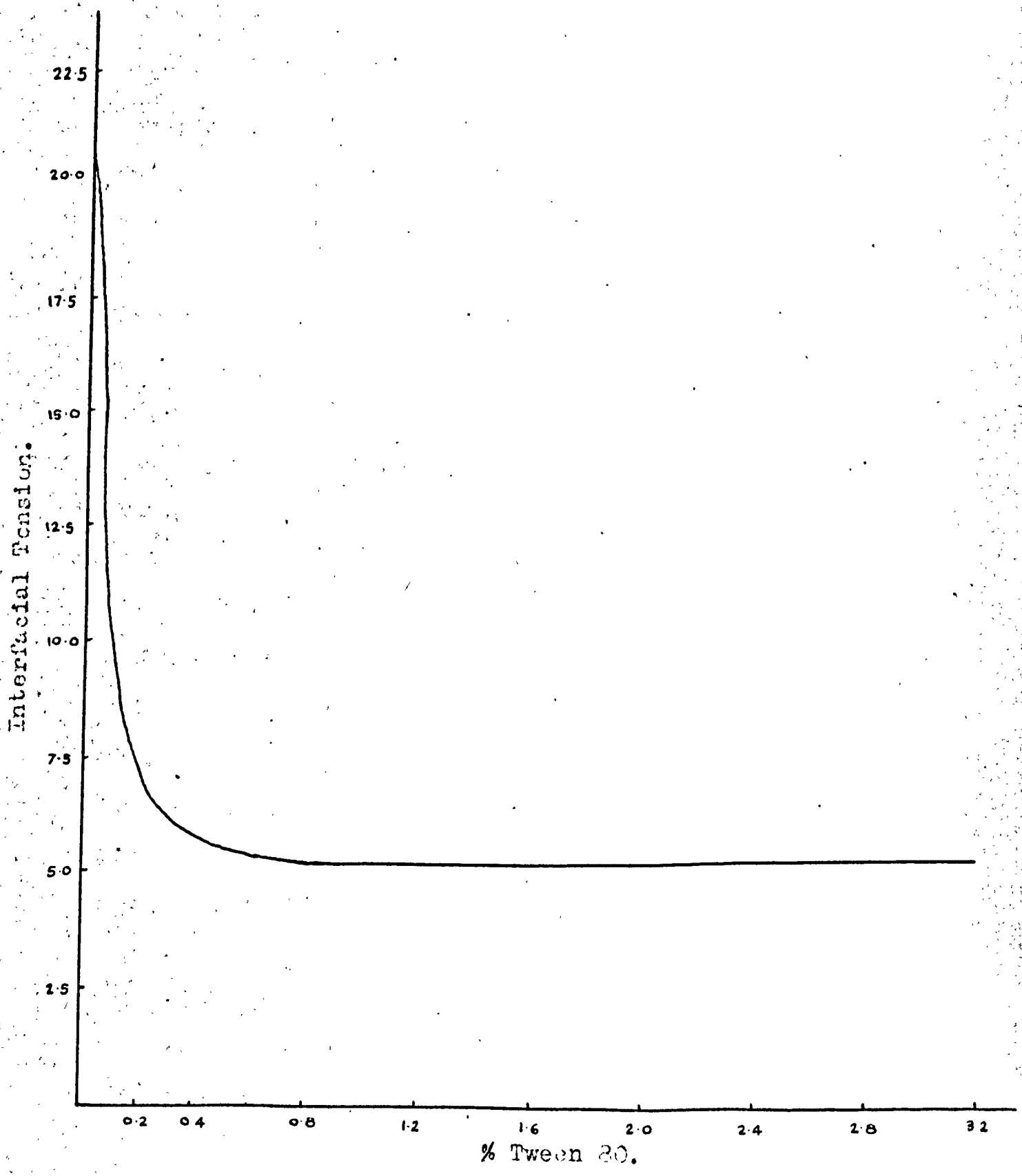


Fig. VII Interfacial Tension between Light Liquid Paraffin and Solutions of Tween 80.

Eisman, Jaconia and Meyer (1953) reported that an aqueous 0.1 per cent Tween 80 solution had no bactericidal action on the spores of Bacillus subtilis. The viability of the spores of Bacillus subtilis in different concentrations of Tween 80 in Ringer's solution is recorded in Tables 57 - 64. No bactericidal action was observed in 7 days in solutions containing up to 0.8 per cent Tween 80. In solution containing 1.6 per cent Tween 80 there were 93 per cent survivors after 7 days,.while in Ringer's solution containing 3.2 per cent Tween 80, only about 40 per cent of the organisms were still viable after 7 days.

Aqueous solutions of Tween 80 in concentrations greater than 0.8 per cent were bactericidal and 0.8 per cent Tween 80 solution exhibited minimal interfacial tension. It was, therefore, decided to employ the solutions of Tween 80 up to the concentration of 0.8 per cent in the study of the relation between interfacial tension and the migration of spores from the oil to aqueous phase.

(iv) The effect of reducing the interfacial tension on the migration of spores of Bacillus subtilis from light liquid paraffin to aqueous phase.

Light liquid paraffin containing a known number of

TABLE 57

THE SURVIVAL OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION CONTAINING 0.05% $^{w}/_{v}$ TWEEN 80.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
0 Hour	90	95	98	86	87	91.2	1.9600	5.6373	39,568,032	
3 Hours	96	86	95	86	86	89.8	1.9533	5.6373	38,960,628	98.5
6 Hours	98	85	86	93	86	89.6	1.9523	5.6373	38,873,856	98.2
24 Hours	83	89	94	85	92	88.6	1.9474	5.6373	38,439,996	97.1
72 Hours	85	87	84	95	94	89.0	1.9494	5.6373	38,613,540	97.6
168 Hours	94	96	86	85	88	89.8	1.9533	5.6373	38,960,628	98.5

TABLE 58

THE SURVIVAL OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION CONTAINING 0.1% $^{W}/_{V}$ TWEEN 80.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
0 Hour	81	74	70	78	71	74.8	1.8739	5.6373	32,452,728	
3 Hours	74	74	85	76	74	77.6	1.8899	5.6373	33,667,536	103.8
6 Hours	76	78	67	68	75	72.8	1.8621	5.6373	31,585,008	97.3
24 Hours	78	78	83	70	70	75.8	1.8797	5.6373	32,886,588	101.3
72 Hours	74	79	70	80	81	76.8	1.8854	5.6373	33,320,448	102.7
168 Hours	70	82	79	70	70	74.2	1.8704	5.6373	32,192,412	99.2

TABLE 59

THE SURVIVAL OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION CONTAINING 0.2% $^{w}/_{v}$ TWEEN 80.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
0 Hours	90	101	90	99	98	95.6	1.9805	5.6373	41,477,016	
3 Hours	91	96	101	101	90	95.6	1.9805	5.6373	41,477,016	100.1
6 Hours	95	97	96	86	88	92.4	1.9657	5.6373	40,088,664	96.7
24 Hours	95	85	84	88	92	88.8	1.9484	5.6373	38,526,768	92.9
72 Hours	96	86	85	95	93	91.0	1.9590	5.6373	39,481,260	95.2
168 Hours	85	96	95	96	85	91.2	1.9600	5.6373	39,568,032	95.4

TABLE 60

THE SURVIVAL OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION CONTAINING 0.4% $^{w}/_{v}$ TWEEN 80.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
0 Hour	74	85	77	85	78	79.8	1.9020	5.6373	34,622,028	
3 Hours	85	78	74	81	75	79.2	1.8987	5.6373	34,361,712	99.3
6 Hours	77	82	77	85	84	79.8	1.9020	5.6373	34,622,028	100.0
24 Hours	83	76	74	84	74	78.2	1.8932	5.6373	33,927,852	98.0
72 Hours	84	73	78	81	82	79.6	1.9009	5.6373	34,535,256	99.8
168 Hours	84	84	74	73	84	79.8	1.9020	5.6373	34,622,028	100.0

TABLE 61

THE SURVIVAL OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION CONTAINING 0.8% $^{W}/_{v}$ TWEEN 80.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
0 Hour	80	82	89	86	90	86.2	1.9355	5.6373	37,398,732	
3 Hours	85	88	80	80	90	84.6	1.9274	5.6373	36,704,556	98.2
6 Hours	80	79	90	87	81	83.4	1.9212	5.6373	36,183,924	96.8
24 Hours	86	81	76	85	80	81.6	1.9117	5.6373	35,402,976	94.7
72 Hours	88	80	81	90	80	83.8	1.9232	5.6373	36,357,468	97.2
168 Hours	86	87	88	80	76	83.4	1.9212	5.6373	36,183,924	96.8

TABLE 62

THE SURVIVAL OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION CONTAINING 1.6% $^{w}/_{v}$ TWEEN 80.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
0 Hours	89	79	78	80	89	83.0	1.9191	5.6373	36,010,380	
3 Hours	78	87	85	80	80	82.0	1.9138	5.6373	35,576,520	98.9
6 Hours	77	76	84	86	85	81.6	1.9117	5.6373	35,402,976	98.3
24 Hours	80	77	87	87	79	82.0	1.9138	5.6373	35,576,520	98.9
72 Hours	81	73	85	77	81	80.0	1.9031	5.6373	34,708,800	96.5
168 Hours	83	84	74	74	73	77.6	1.8899	5.6373	33,667,536	93.5

TABLE 63

THE SURVIVAL OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION CONTAINING 3.2% $^{W}/_{v}$ TWEEN 80.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	No. of organisms per ml. of suspension	Percentage Survival
0 Hours	85	75	78	84	84	81.4	1.9106	5.6373	35,316,204	
3 Hours	79	86	78	87	76	81.2	1.9096	5.6373	35,229,432	99.8
6 Hours	85	76	77	84	84	81.2	1.9096	5.6373	35,229,432	99.8
24 Hours	80	70	73	80	77	76.0	1.8808	5.6373	32,973,360	93.4
72 Hours	42	49	49	40	49	45.8	1.6609	5.6373	19,870,788	56.3
168 Hours	30	34	36	30	36	33.2	1.5211	5.6373	14,404,152	40.8

TABLE 64

THE SURVIVAL OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION CONTAINING DIFFERENT CONCENTRATIONS OF TWEEN 80.

Concentration of Tween 80 in Ringer's solution % w/v	Percentage survival after storage.					
	0 Hour	3 Hours	6 Hours	24 Hours	72 Hours	168 Hours
Nil	100.0	101.0	101.2	101.0	99.6	98.4
0.05	100.0	98.5	98.2	97.1	97.6	98.5
0.1	100.0	103.8	97.3	101.3	102.7	99.2
0.2	100.0	100.0	96.7	92.9	95.2	95.4
0.4	100.0	99.3	100.0	98.0	99.8	100.0
0.8	100.0	98.2	96.8	94.7	97.2	96.8
1.6	100.0	98.9	98.3	98.9	96.5	93.5
3.2	100.0	99.8	99.8	93.4	56.3	40.8

dry "uncoated" spores of Bacillus subtilis was floated over Ringer's solution containing different concentrations of Tween 80. The aqueous phase was collected from separators after predetermined intervals of time and the percentage of spores passing from the oil into the aqueous phase during each time period was calculated (Tables 65 - 71 and Fig. VIII). The percentage of spores passing through the interface to the aqueous phase increased as the concentration of Tween 80 in Ringer's solution increased. Only about 14 per cent of the spores migrated from the oil to the Ringer's solution containing nil Tween 80 in 72 hours, whereas in the same time about 57 per cent of the spores migrated from the oil to the Ringer's solution containing 0.8 per cent Tween 80. During the same period most of the spores must have sedimented through the oil to the interface (Table 56).

The above experiment indicated that there is a linear relationship between the interfacial tension and the percentage of spores passing through the interface from the oil to the aqueous phase (Fig IX). The experiment also suggested that if it had been possible to reduce sufficiently the interfacial tension by a non-bactericidal surface-active agent, it should have been possible to obtain all the organisms from the oil into the aqueous phase. Higher concentra-

tions of Tween 80 did not reduce further the interfacial tension and, moreover, were bactericidal. Thus, it was not possible to study further the effect of interfacial tension on the migration of spores of Bacillus subtilis from the oil to the aqueous phase.

TABLE 65

MIGRATION OF SPORES OF BACILLUS SUBTILIS FROM LIGHT LIQUID PARAFFIN INTO RINGER'S SOLUTION CONTAINING DIFFERENT CONCENTRATIONS OF TWEEN 80.

Sedimentation Time :- 1½ Hours.

Initial number of organisms in the oil	Concentration of Tween 80 in Ringer's solution % w/v	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Total number of organisms obtained in aqueous phase	Percentage of initial number of organisms
95,308,800	Nil	280	292	301	281	286	288.0	2.4594	3.8627	2,099,568	2.2
	0.05	398	410	400	388	416	402.4	2.6046	3.8627	2,933,564	3.07
	0.1	262	254	240	269	255	256.0	2.4082	4.1596	3,695,731	3.88
	0.2	318	332	340	320	333	328.6	2.5167	4.1596	4,743,817	4.97
	0.4	409	400	422	401	419	410.2	2.1683	4.1596	5,921,832	6.21
	0.8	443	440	464	459	446	450.6	2.6538	4.1596	6,505,064	6.82

TABLE 66

MIGRATION OF SPORES OF BACILLUS SUBTILIS FROM LIGHT LIQUID PARAFFIN INTO RINGER'S SOLUTION CONTAINING DIFFERENT CONCENTRATIONS OF TWEEN 80.

Sedimentation Time :- 3 Hours.

Initial number of organisms in the oil	Concentration of Tween 80 in Ringer's solution % W/v	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Total number of organisms obtained in aqueous phase	Percentage of initial number of organisms
95,308,800	Nil	72	66	68	63	74	70.6	1.8488	4.8560	5,068,978	5.32
	0.05	88	95	85	88	96	90.4	1.9562	4.8560	6,490,590	6.81
	0.1	116	119	106	108	116	113.0	2.0531	4.8560	8,113,238	8.51
	0.2	130	144	139	136	135	134.8	2.1297	4.8560	9,678,446	10.15
	0.4	150	165	161	154	153	156.6	2.1948	4.8560	11,243,655	11.8
	0.8	166	185	170	182	178	176.2	2.2460	4.8560	12,650,907	13.3

TABLE 67

MIGRATION OF SPORES OF BACILLUS SUBTILIS FROM LIGHT LIQUID PARAFFIN INTO RINGER'S SOLUTION CONTAINING DIFFERENT CONCENTRATIONS OF TWEEN 80.

Sedimentation Time :- 6 Hours.

Initial number of organisms in the oil	Concentration of Tween 80 in Ringer's solution % w/v	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Total number of organisms obtained in aqueous phase	Percentage of initial number of organisms
95,308,800	Nil	90	85	9[illegible]	98	9[illegible]	93.8	1.9722	4.8560	6,734,705	7.07
	0.05	120	120	108	123	121	118.4	2.0734	4.8560	8,500,950	8.92
	0.1	145	156	156	140	156	150.6	2.1778	4.8560	10 812,863	11.35
	0.2	204	206	203	187	204	200.8	2.3027	4.8560	14 417,151	15.13
	0.4	240	235	240	249	258	244.4	2.3881	4.8560	17,547,569	18.41
	0.8	269	256	274	260	252	265.8	2.4213	4.8560	18,940,460	19.87

TABLE 68

MIGRATION OF SPORES OF BACILLUS SUBTILIS FROM LIGHT LIQUID PARAFFIN INTO RINGER'S SOLUTION CONTAINING DIFFERENT CONCENTRATIONS OF TWEEN 80.

Sedimentation Time :- 24 Hours.

Initial number of organisms in the oil	Concentration of Tween 80 in Ringer's solution % W/V	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Total number of organisms obtained in aqueous phase	Percentage of initial number of organisms
95,308,800	Nil	24	28	28	23	24	25.4	1.4048	5.6473	11,274,677	11.83
	0.05	41	46	47	41	46	44.2	1.6454	5.6473	19,620,005	20.58
	0.1	35	31	30	30	35	32.2	1.5079	5.9448	28,359,166	29.75
	0.2	38	41	42	42	36	39.8	1.5999	5.9448	35,052,633	36.77
	0.4	41	47	46	43	47	44.8	1.6513	5.9448	39,456,230	41.39
	0.8	48	44	45	50	50	47.4	1.6758	5.9448	41,639,252	43.69

TABLE 69

MIGRATION OF SPORES OF BACILLUS SUBTILIS FROM LIGHT LIQUID PARAFFIN INTO RINGER'S SOLUTION CONTAINING DIFFERENT CONCENTRATIONS OF TWEEN 80.

Sedimentation time :- 72 Hours.

Initial number of organisms in the oil	Concentration of Tween 80 in Ringer's solution % W/V	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Total number of organisms obtained in aqueous phase	Percentage of initial number of organisms
95,308,800	Nil	15	17	14	16	14	15.2	1.1818	5.9448	13,386,935	14.05
	0.05	38	33	36	38	38	36.6	1.5635	5.9448	32,234,331	33.82
	0.1	50	49	45	47	44	47.0	1.6721	5.9448	41,393,813	43.43
	0.2	60	56	54	54	59	56.6	1.7528	5.9448	49,848,720	52.30
	0.4	64	60	60	58	56	59.6	1.7752	5.9448	52,490,878	55.08
	0.8	65	59	58	63	64	61.8	1.7910	5.9448	54,437,353	57.12

TABLE 70

MIGRATION OF SPORES OF BACILLUS SUBTILIS FROM LIGHT LIQUID PARAFFIN INTO RINGER'S SOLUTION CONTAINING DIFFERENT CONCENTRATIONS OF TWEEN 80.

Sedimentation time :- 168 Hours

Initial Number of organisms in the oil	Concentration of Tween 80 in Ringer's solution % w/v	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Total No. of organisms obtained in aqueous phase	Percentage of initial number of organisms
95,308,800	Nil	15	18	14	14	15	15.2	1.1818	5.9448	13,386,935	14.05
	0.05	38	33	39	38	38	37.2	1.5705	5.9448	32,762,784	34.38
	0.1	50	44	49	49	45	47.2	1.6739	5.9448	41,746,101	43.78
	0.2	57	62	63	65	61	61.6	1.7896	5.9448	54,252,317	56.92
	0.4	67	63	62	66	60	63.6	1.8035	5.9448	56,013,756	58.77
	0.8	68	62	63	63	69	65.0	1.8129	5.9448	57,262,351	60.08

TABLE 71

EFFECT OF TWEEN 80 ON RATE OF MIGRATION OF SPORES OF BACILLUS SUBTILIS FROM LIGHT LIQUID PARAFFIN INTO AQUEOUS PHASE.

Concentration of Tween 80 in Ringer's solution % w/v	Percentage of organisms initially in oil obtained in aqueous phase after contact between two phases.					
	1½ Hours	3 Hours	6 Hours	24 Hours	72 Hours	168 Hours
Nil	2.2	5.32	7.07	11.83	14.05	14.05
0.05	3.07	6.81	8.92	20.58	33.82	34.38
0.1	3.88	8.51	11.35	29.75	43.43	43.78
0.2	4.97	10.15	15.13	36.77	52.30	56.92
0.4	6.21	11.8	18.41	41.39	55.08	58.77
0.8	6.82	13.3	19.87	43.69	57.12	60.08

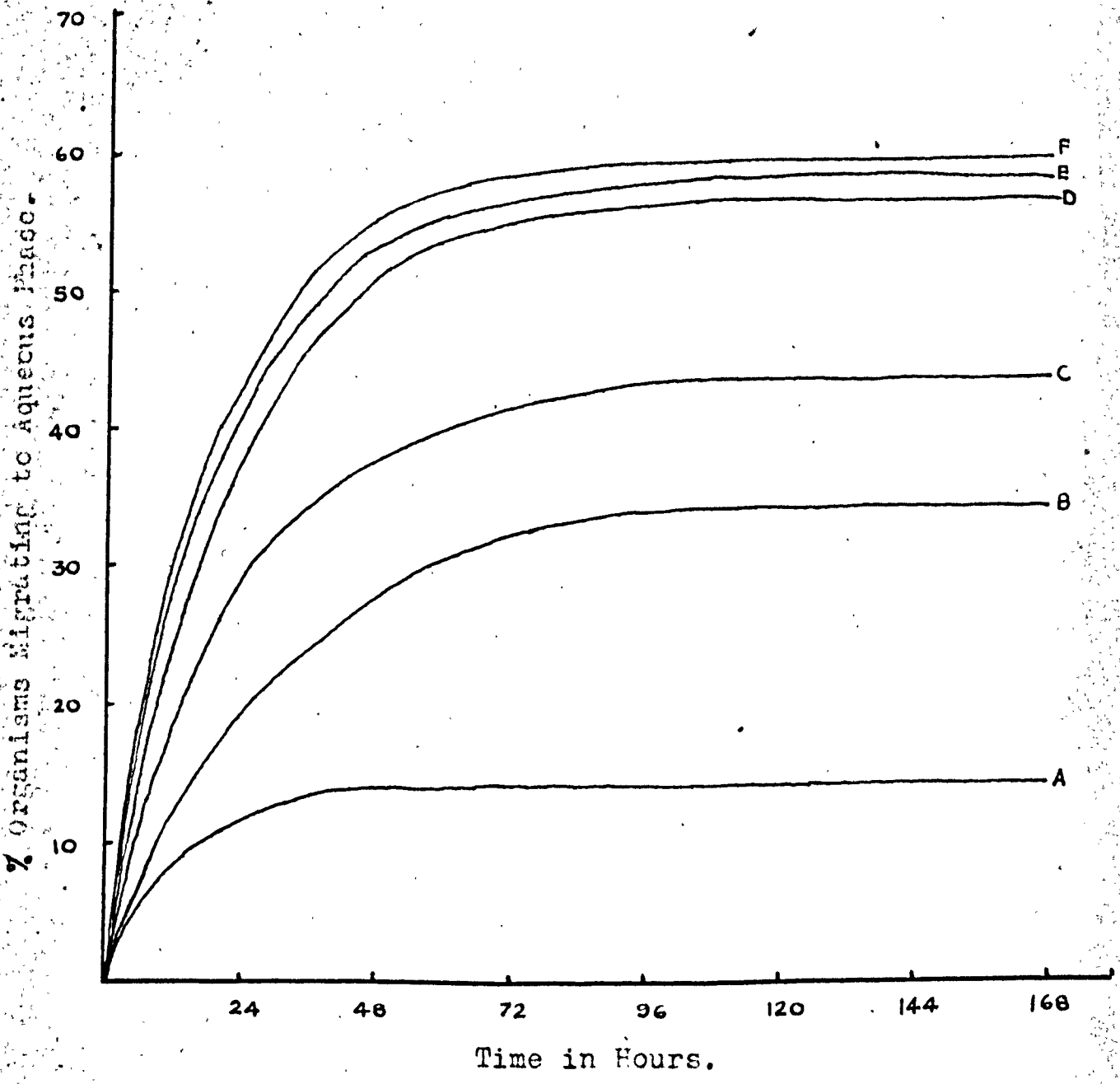


Fig. VIII Migration of Viable Spores of Bacillus subtilis from Light Liquid Paraffin to Solutions of Tween 80 in Ringer's Solution.

A:- Ringer's solution.
B:- Ringer's solution containing 0·05% Tween 80.
C:- Ringer's solution containing 0·1 % Tween 80.
D:- Ringer's solution containing 0.2 % Tween 80.
E:- Ringer's solution containing 0.4 % Tween 80.
F:- Ringer's solution containing 0.8 % Tween 80.

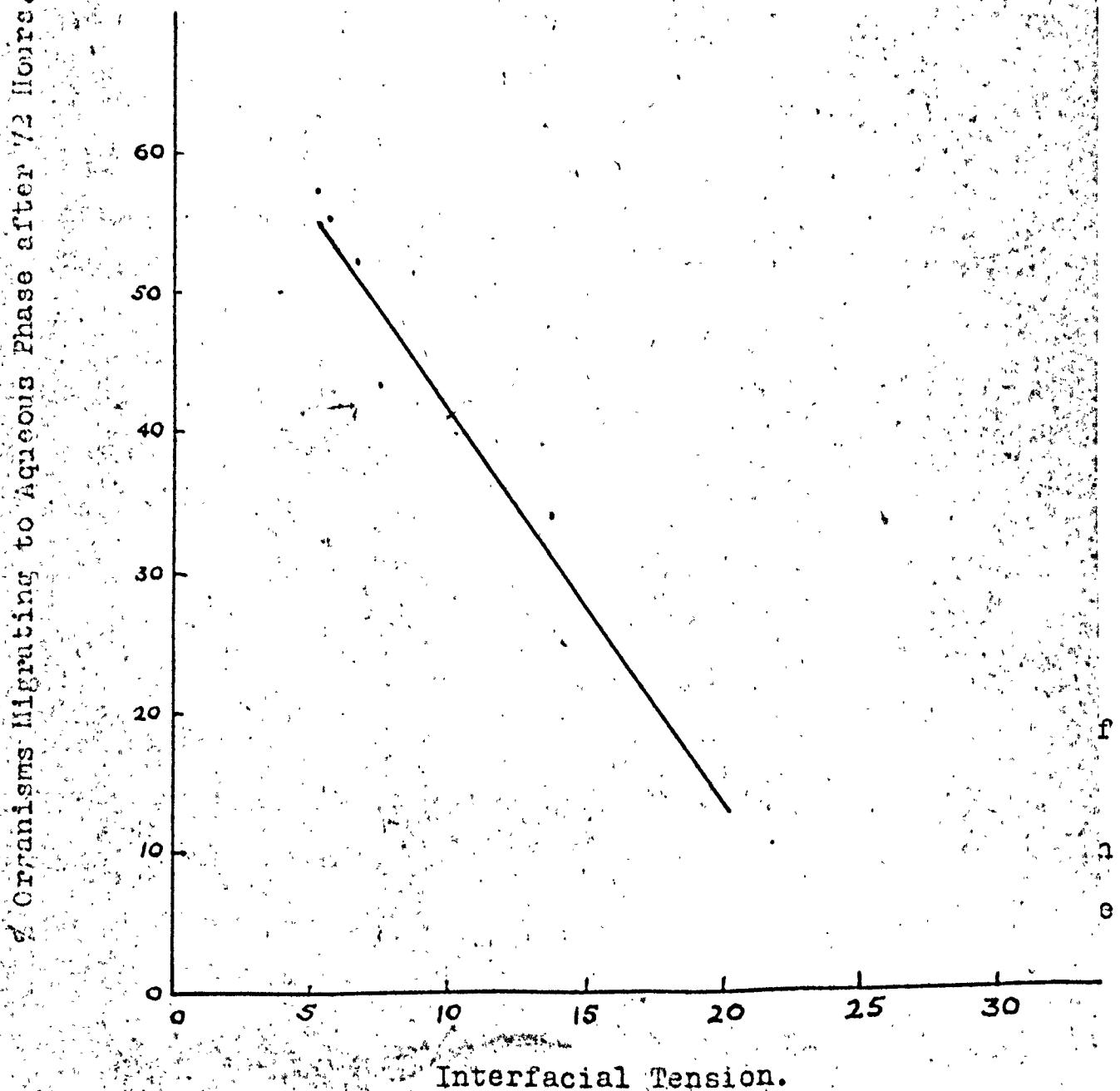


Fig. IX Relationship between Interfacial Tension and Percentage of Initial Number of Organisms Obtained in Aqueous Phase.

(E) THE ENUMERATION OF VIABLE SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN BY A CENTRIFUGING METHOD.

The migration experiments (page 165) indicated that when light liquid paraffin infected with dry "uncoated" spores of Bacillus subtilis was floated over sterile Ringer's solution, about 16 per cent of the organisms migrated from the oil to the Ringer's solution, while the majority of them collected at the interface. The ability of some of the organisms to penetrate into the Ringer's solution by virtue of their momentum, suggested that if all the organisms in the oil could be collected in the Ringer's solution by increasing their momentum, it might be possible to use the count in the Ringer's solution as an estimate of the number of viable organisms originally in the oil. Such a method would obviate the use of bactericidal organic solvents which are used in the technique of Bullock and Keepe (1951).

The sedimentation of dry "uncoated" spores through the oil was extremely slow. It would not have been practicable to prolong the sedimentation time to permit all the spores to sediment through the oil, because during this prolonged time some of the spores might have been killed by the oil. To accelerate the transfer of the organisms from the oil to the Ringer's solution, the system was centrifuged at high speed.

The increased velocity produced by centrifuging endowed the organisms with sufficient momentum to pass through the interface into the Ringer's solution.

The effect of variation in centrifuging time at approximately 8300 g., was determined by floating 0.52 millilitre of oil infected with dry "uncoated" spores of Bacillus subtilis over 2 millilitres of sterile Ringer's solution in a series of gyro-centrifuge tubes of 20 millilitres capacity. Assuming the applicability of Stoke's law, the time required for single spores to sediment through 1.0 millimetre layer of oil at 8300 g., was calculated to be about 60 minutes. Centrifuge tubes containing infected oil and Ringer's solution were centrifuged for 5, 10, 20, 30 or 60 minutes, four replicate tubes being employed for each time (Table 72). The counts on the Ringer's solution indicated that there was no significant difference between the number of organisms obtained in the Ringer's solution after centrifuging for 5, 10, 20, 30 or 60 minutes. This suggested that all the organisms originally in the oil had penetrated into the Ringer's solution after centrifuging at 8300 g., for not more than 5 minutes. The migration experiments had shown that the infected oil consisted of aggregates of varied sizes. During centrifuging the smaller aggregates undoubtedly came into contact with the rapidly

TABLE 72

RELATIONSHIP BETWEEN TIME OF CENTRIFUGING INFECTED LIGHT LIQUID PARAFFIN-RINGER'S SOLUTION SYSTEM AND VIABLE COUNT OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION.

Expected number of organisms :- 43,911,500/ ml. of oil.

Time of Centri-fuging	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilu-tion Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
5 Minutes	No.1.	82	88	88	94	94	88.5	1.9469	5.7024	44,607,500	101.59
	No.2.	88	85	96	90	84					
	No.3.	90	90	98	86	90					
	No.4.	92	81	84	80	90					
10 Minutes	No.1.	92	80	80	79	82	85.1	1.9299	5.7024	42,893,800	97.68
	No.2.	90	92	81	82	80					
	No.3.	92	94	82	88	90					
	No.4.	78	90	81	80	89					

TABLE 72 Cont'd.

Time of Centrifuging	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
20 Minutes	No.1.	77	84	88	80	87	86.25	1.9358	5.7024	43,473,400	99.00
	No.2.	83	81	93	92	88					
	No.3.	88	80	80	90	93					
	No.4.	93	83	94	90	81					
30 Minutes	No.1.	91	80	88	81	83	85.9	1.9340	5.7024	43,297,000	98.6
	No.2.	90	80	79	89	82					
	No.3.	96	90	84	90	83					
	No.4.	86	92	80	91	83					
60 Minutes	No.1.	96	84	92	82	82	88.2	1.9455	5.7024	44,456,300	101.24
	No.2.	80	92	79	79	93					
	No.3.	90	90	87	100	99					
	No.4.	87	95	80	83	94					

moving larger aggregates, adhered to them, and thus were carried down into the Ringer's solution at the same velocity as that of the larger aggregates. To allow for the possible contingency that some comparatively small aggregates of organisms might be in the oil, the centrifuging time was fixed at 30 minutes.

After centrifuging the two phases at 8300 g., for 30 minutes, the upper oily layer was pipetted off. Viable counts revealed that if the interface was disturbed and removed with the layer of oil about 12 per cent of the organisms were lost. This loss, which resulted from the removal of the interface, could not be reduced by replacing the Ringer's solution by Ringer's solution containing 0.8 per cent Tween 80. Thus, a proportion of the organisms did not penetrate the interface and pass into the Ringer's solution even when the system was centrifuged at 8300 g., and the interfacial tension reduced by the inclusion of the Tween 80 in the Ringer's solution. Great care was, therefore, taken not to disturb the interface while removing the oil.

Bullock and Keepe (1951) have shown that the technique developed by them, for estimating the number of viable spray-dried organisms in a sample of oil, can give a count which deviates from an expected value only by an amount which can be attributed to random sampling errors. Hence, the

reliability of the proposed centrifuging method was compared with that developed by Bullock and Keepe (1951).

For the purpose of this comparison a viable count was performed initially on the contents of a tube containing freeze-dried spores of Bacillus subtilis. The count of the viable organisms in this tube of dried material was regarded as representative of the count of the remaining tubes of the same batch of freeze-dried material, since it has been shown previously (Table 38) that there is no significant difference between the number of viable organisms in the freeze-dried tubes of the same batch. The contents of a second similar tube from the same batch were added to 25 millilitres of light liquid paraffin and shaken vigorously. Viable counts were performed on 0.52 millilitre (i.e., 50 drops) samples of the infected oil by Bullock and Keepe's method. At the same time 0.52 millilitre samples of the infected oil were floated over 2 millilitres of sterile Ringer's solution in centrifuge tubes and centrifuged immediately at 8300 g. for 30 minutes. After centrifuging, the oil was pipetted off without disturbing the interface and the organisms deposited in the Ringer's solution were resuspended in the Ringer's solution, the volume made up to 3 millilitres and viable counts were performed on the suspensions.

Table 73 records that in 2 duplicate experiments 100.27 and 100.24 per cent of the expected number of organisms were recorded in the oil by Bullock and Keepe's method (1951), whereas 100.62 and 103.64 per cent of the expected number of organisms were recovered by the centrifuging method. Thus the centrifuging method of performing viable counts on an oil containing spores of Bacillus subtilis was at least as satisfactory as the method of Bullock and Keepe. Further, the centrifuging method possessed the advantage of avoiding the use of organic solvents which may have a lethal effect on organisms less resistant than the strain of Bacillus subtilis used. The centrifuging method was also much simpler in manipulation than the method of Bullock and Keepe. It was, therefore, decided to employ this method for counting viable spores in oil simultaneously with Bullock and Keepe's method, so as to obtain more data regarding its usefulness and experience in manipulation.

An Analysis of Variance (Table 74-75) of the counts performed by each method indicated that the two methods were equally capable of determining viable organisms in oil. The variation in the counts, given by both methods, can be attributed to random sampling errors. The probabilities of obtaining the variance ratio recorded in Tables 74-75 are lower than those recorded previously for a count performed by

TABLE 73

ENUMERATION OF VIABLE SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN BY BOTH METHODS.

Experiment		Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes	Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml of oil obtained	Percentage of expected number of organisms obtained
I	Bullock and Keepe's Method	338,387,950	No.1.	120 126 124 132 120	121.76	1.0852	6.4446	339,029,950	100.20
			No.2.	122 116 128 113 112					
			No.3.	120 115 106 120 121					
			No.4.	126 132 120 123 126					
			No.5.	127 133 123 120 119					
	Centrifuging Method	338,387,950	No.1.	132 146 146 134 140	136.0	2.1335	6.3984	340,484,400	100.62
			No.2.	137 139 143 129 128					
			No.3.	122 134 131 123 136					
			No.4.	146 144 143 133 134					

TABLE 73 Cont'd.

Experiment		Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
II	Bullock and Keepe's Method	381,363,480	No.1.	60	64	57	53	58	55.88	1.7472	6.8491	[illegible]	101.40
			No.2.	54	59	50	58	56					
			No.3.	51	52	49	54	56					
			No.4.	53	50	59	58	56					
			No.5.	60	55	62	60	53					
	Centrifuging Method	381,363,480	No.1.	88	76	85	76	90	79.0	1.8976	6.6992	395,225,900	103.64
			No.2.	80	68	76	79	68					
			No.3.	75	89	76	82	88					
			No.4.	84	74	71	85	71					

Bullock and Keepe's method (Table 45). This could be attributed to the fact that the suspension used for the counts recorded in Table 43 had been lightly centrifuged to deposit any larger aggregates present in the suspension. The suspension used for the comparison of the two methods for performing viable counts had not been initially centrifuged, because it was necessary to know the expected number of organisms in a sample of the suspension. The oil was, therefore, more heterogeneous than the lightly centrifuged suspension.

TABLE 74

ANALYSIS OF VARIANCE OF COUNTS OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN BY THE METHOD OF BULLOCK AND KEEPE.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
I	(a) Between samples	343	4	85.75	2.663	0.05-0.1
	(b) Within samples	644	20	32.2		
	Total	987	24			
II	(a) Between samples	118	4	29.5	2.278	0.05-0.1
	(b) Within samples	259	20	12.95		
	Total	377	24			

TABLE 75

ANALYSIS OF VARIANCE OF COUNTS OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN BY CENTRIFUGING METHOD.

Experiment	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
I	(a) Between samples	392	3	130.66	3.172	0.05-0.1
	(b) Within samples	659	16	41.19		
	Total	1051	19			
II	(a) Between samples	270	3	90.0	1.755	0.1-0.2
	(b) Within samples	825	16	51.31		
	Total	1095	19			

(F) IMPROVEMENT OF THE UNIFORMITY OF A SUSPENSION OF DRY "UNCOATED" SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

The rate of sedimentation of spores of Bacillus subtilis through light liquid paraffin led to the conclusion that the oily suspension consisted of aggregates of varied sizes. However, 0.52 millilitre samples of the suspension were found to contain equal numbers of viable organisms, in spite of the heterogeneity of the suspension. Clearly the concentration of the organisms was so high that the heterogeneous nature of the suspension was concealed. The use of much smaller samples might have revealed the non-uniformity of the suspension but it would have considerably increased the sampling error. The alternative to the use of small samples to reveal the heterogeneity of the suspension would be the use of a much more dilute suspension. This latter technique was adopted because it avoided the large sampling errors which are unavoidably associated with small samples.

The oily suspension of dry "uncoated" spores of Bacillus subtilis was serially diluted with further volumes of sterile light liquid paraffin and viable counts were performed, by both the method of Bullock and Keepe and the centrifuging

method, on samples taken from each dilution (Tables 76-77).

The centrifuging method usually gave a higher count than did the method of Bullock and Keepe. In order that the counts obtained by the two methods could be compared at different dilutions of the suspension (i.e. different inoculum sizes), the counts obtained were converted to percentages of the number of organisms expected in different dilutions. The percentages of the expected numbers given by the two methods were compared by a simple "t" test (Table 78), which revealed that, although the centrifuging method did normally give a higher count, the difference between the counts obtained by the two methods was not significant. The difference in the viable counts given by the two methods can be attributed to the loss of some organisms by floatation in the method of Bullock and Keepe. Bullock and Booth (1953) stated that the particles of a spray-dried powder have the form of whole or broken hollow shells some of which retain air. The latter authors believed that on centrifuging a sample of infected oil in petroleum ether some of the peptone particles carrying adherent organisms could rise to the surface and thus be lost on decantation. The freeze-dried material used in the present experiments contained light flaky particles, and thus it is probable that a loss was incurred by a similar mechanism in the count by the method of Bullock and Keepe. Such

TABLE 76

DILUTION OF UNFILTERED SUSPENSION OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

(Method of Bullock and Keepe).

Dilution	Expected number of organisms per ml. of oil.	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
Initial		No.1.	38	35	32	38	38	35.92	1.5553	6.1440	50,049,527	100.00
		No.2.	41	39	31	41	40					
		No.3.	30	35	33	38	30					
		No.4.	37	39	30	37	30					
		No.5.	40	32	36	39	39					
(18.3) fold	2,734,946	No.1.	87	92	98	99	88	97.6	1.9894	4.4411	2,695,224	98.55
		No.2.	107	96	105	95	104					
		No.3.	94	99	96	85	96					
		No.4.	98	98	96	108	109					
		No.5.	104	104	95	90	97					

TABLE 76 Cont'd.

Dilution	Expected number of organisms per ml. of oil.	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
$(18.3)^2$ fold	149,450	No.1.	52	40	54	50	50	51.88	1.7150	3.3651	150,258	100.54
		No.2	60	45	50	49	46					
		No.3.	44	52	49	42	50					
		No.4.	68	58	56	55	57					
		No.5.	57	50	52	61	50					
$(18.3)^3$ fold	8166	No.1.	48	51	50	40	48	51.28	1.7100	2.2068	8256	101.1
		No.2.	56	50	52	50	50					
		No.3.	52	44	48	48	54					
		No.4.	49	48	40	50	46					
		No.5.	69	57	64	60	58					

TABLE 76 Cont'd.

Dilution	Expected number of organisms per ml. of oil.	Sample	Counts of 5-replicate tubes			Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained.
		No.1.	44	42	46					
		No.2.	26	30	30					
		No.3.	60	59	57					
		No.4.	42	38	40					
$(18.4)^4$ fold	446	No.5.	32	35	28	42.0	1.6232	0.9823	404	90.6
		No.6.	32	38	38					
		No.7.	50	55	50					
		No.8.	35	40	36					
		No.9.	39	41	46					
		No.10.	46	52	53					

TABLE 77

DILUTION OF UNFILTERED SUSPENSION OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

(Centrifuging method).

Time of centrifuging :- 30 minutes.

Dilution	Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
Initial	50,049,527	No.1.	96	109	97	99	101	101.5	1.6085	5.7024	51,150,214	102.3
		No.2.	104	95	96	107	96					
		No.3.	114	114	104	100	102					
		No.4.	105	101	96	93	101					
(18.3) fold	2 ,734,946	No.1.	70	76	74	70	66	70.7	1.8494	4.6172	2,929,093	107.1
		No.2.	74	70	70	76	66					
		No.3.	61	70	70	68	60					
		No.4.	80	80	66	71	76					

TABLE 77 Cont'd.

Dilution	Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained.
$(18.3)^2$ fold	149,450	No.1.	68	81	76	71	68	72.5	1.8603	3.3230	152,491	102.0
		No.2.	79	90	88	80	75					
		No.3.	60	70	69	70	65					
		No.4.	60	75	72	63	70					
$(18.3)^3$ fold	8166	No.1.	135	122	134	130	130	122.3	2.0885	2.4639	8450	103.5
		No.2.	110	121	116	110	112					
		No.3.	129	141	137	132	139					
		No.4.	115	104	114	109	106					

TABLE 77 Cont'd.

Dilution	Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained.
$(18.3)^4$ fold	446	No.1.	20	20	23	22	24	24.72	1.2931	1.2405	427	95.8
		No.2.	22	20	16	16	16					
		No.3.	20	23	21	19	20					
		No.4.	23	26	28	28	28					
		No.5.	36	40	35	38	39					
		No.6.	28	26	22	22	22					
		No.7.	26	23	22	26	28					
		No.8.	26	23	26	26	21					

TABLE 78

THE DIFFERENCE BETWEEN THE TWO METHODS FOR COUNTING BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

Dilutions	Bullock and Keepe's Method (X_A)	Centrifuging Method (X_B)	$X_B - X_A$	X^2 $(X_B - X_A)^2$	$= S\sqrt{\frac{N}{N-1}}$	$t = \frac{(X - \bar{X})\sqrt{N-1}}{S}$	P
Initial	100.00	102.30	2.30	5.29			
(18.3) fold	98.55	107.10	8.55	73.10			
$(18.3)^2$ fold	100.54	102.00	1.46	2.13	10.12	0.799	0.4-0.5
$(18.3)^3$ fold	101.1	103.50	2.40	5.76			
$(18.3)^4$ fold	90.6	95.80	4.20	17.64			
			$\therefore \bar{X} = 3.78$	$\therefore S^2 \underline{X}^2 - \bar{X}^2 = 89.64$			

a loss would result in too low an estimate of the number of organisms originally present in the oil.

In can be concluded that the centrifuging method was at least as accurate as the method of Bullock and Keepe for counting spores of Bacillus subtilis in the oil. Since it possesses the advantages described above, it is suggested as a preferred method for counting viable spores in oil.

An Analysis of Variance of the counts obtained by the two methods (Tables 79-80) indicated that equal volumes of diluted suspensions contained unequal numbers of viable organisms. As the dilution of the infected oil progressed, the "between sample" variation became significantly greater than the "within sample" variation. This confirmed the belief that a suspension of spores of Bacillus subtilis in oil was heterogeneous and contained aggregates of varied sizes rather than discrete individual organisms.

If the larger aggregates of adhering organisms in the suspension could be removed it would be possible to reduce the "between aggregates size" variation and thereby improve the uniformity of the viable counts in the different samples.

An attempt was made to remove the larger aggregates by filtration of the suspension through a No.4 sintered glass filter with an approximate pore size of 35 microns.

TABLE 78

ANALYSIS OF VARIANCE OF SAMPLES OF DIFFERENT DILUTIONS OF UNFILTERED SUSPENSION OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

(Bullock and Keepe's method).

Dilution	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
Initial	(a) Between samples	125	4	31.25	1.722	0.1-0.2
	(b) Within samples	363	20	18.15		
	Total	488	24			
(18.3) fold	(a) Between samples	341	4	85.25	2.595	0.05-0.1
	(b) Within samples	657	20	32.85		
	Total	998	24			
$(18.3)^2$ fold	(a) Between samples	416	4	104.00	3.955	0.01-0.05
	(b) Within samples	526	20	26.30		
	Total	942	24			
$(18.3)^3$ fold	(a) Between samples	739	4	184.75	12.568	<0.001
	(b) Within samples	294	20	14.70		
	Total	1033	24			
$(18.3)^4$ fold	(a) Between samples	2383	9	264.78	32.095	<0.001
	(b) Within samples	165	20	8.25		
	Total	2548	29			

TABLE 80

ANALYSIS OF VARIANCE OF SAMPLES OF DIFFERENT DILUTIONS OF UNFILTERED SUSPENSION OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

(Centrifuging Method).

Dilution	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
Initial	(a) Between samples	191	3	63.66	1.478	>0.2
	(b) Within samples	689	16	43.06		
	Total	880	19			
(18.3) fold	(a) Between samples	192	3	64.00	1.89	0.1-0.2
	(b) Within samples	520	16	33.87		
	Total	712	19			
$(18.3)^2$ fold	(a) Between samples	646	3	215.33	2.702	0.05-0.1
	(b) Within samples	1275	16	79.69		
	Total	1921	19			
$(18.3)^3$ fold	(a) Between samples	2364	3	788.00	32.25	<0.001
	(b) Within samples	386	16	24.12		
	Total	2750	19			
$(18.3)^4$ fold	(a) Between samples	1178	7	168.29	22.17	<0.001
	(b) Within samples	243	32	7.59		
	Total	1421	39			

The initial suspension before filtration was cloudy and after filtration was clear. The removal of the larger aggregates resulted in a suspension containing only about 3 per cent of the number of organisms originally present in the oil. Thus, it appeared that about 97 per cent of the organisms in the unfiltered suspension probably had dimensions greater than about 35 microns. This observation confirmed the conclusion of the sedimentation experiment (Table 56) that about the same percentage of the organisms were in the form of aggregates having dimensions greater than 34 microns.

Serial dilutions were made of the clear filtered suspension in oil and viable counts were performed on samples from each dilution by both the centrifuging method and by the method of Bullock and Keepe(Tables 81-82). An Analysis of Variance of the counts shows that in each dilution, the variation of "between samples" counts was not significantly greater than the "within sample" variation (Tables 83-84). This result contrasted markedly with that obtained for the unfiltered suspensions, and indicated a much greater uniformity of the distribution of the organisms, even in the very dilute suspension.

More than 90 per cent of the expected number of organisms was obtained in each dilution by the method of

TABLE 81

DILUTION OF FILTERED SUSPENSION OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

(Method of Bullock and Keepe).

Dilution	Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes	Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
Initial		No.1.	154 160 150 165 150	155.0	2.1903	3.7674	907,215	100.0
		No.2.	171 162 154 160 156					
		No.3.	156 146 158 148 150					
		No.4.	145 160 154 156 152					
		No.5.	160 152 148 156 152					
(18.3) fold	49574	No.1.	160 150 144 159 156	148.2	2.1709	2.4886	45646	92.1
		No.2.	146 148 149 136 149					
		No.3.	143 146 144 153 149					
		No.4.	146 140 153 153 152					
		No.5.	153 148 149 140 139					

TABLE 81 (Cont'd.

Dilution	Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml of oil obtained	Percentage of expected number of organisms obtained
$(18.3)^2$ fold	2708	No.1.	68	72	64	69	66	64.2	1.8075	1.5798	2469	91.2
		No.2.	60	68	60	68	68					
		No.3.	62	56	60	59	66					
		No.4.	60	68	68	66	60					
		No.5.	60	60	68	61	68					
$(18.3)^3$ fold	148	No.1.	13	10	13			14	0.1461	0.9823	134	90.6
		No.2.	18	13	19							
		No.3.	14	14	10							
		No.4.	11	14	10							
		No.5.	10	13	14							
		No.6.	16	16	12							
		No.7.	16	14	18							
		No.8.	16	13	16							
		No.9.	13	14	18							
		No.10.	12	16	16							

TABLE 82

DILUTION OF FILTERED SUSPENSION OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

(Centrifuge Method)

Time of Centrifuging :- 30 minutes.

Dilution	Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
Initial	907,215	No.1.	66	74	74	70	67	71.6	1.8549	3.7674	752,989	82.9
		No.2.	84	79	70	74	75					
		No.3.	72	67	78	62	72					
		No.4.	66	65	73	70	74					
(18.3) fold	49574	No.1.	78	76	68	66	72	73.2	1.8645	2.7427	40516	81.7
		No.2.	78	80	72	80	72					
		No.3.	70	70	72	68	80					
		No.4.	70	78	75	70	72					

TABLE 82 Cont'd.

Dilution	Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained.
$(18.3)^2$ fold	2708	No.1.	70	73	70	75	70	74.8	1.8739	1.4314	2067	76.4
		No.2 .	70	80	71	80	70					
		No.3.	81	83	71	79	77					
		No.4.	78	79	70	79	70					
$(18.3)^3$ fold	148	No.1.	7	8	6	5	8	6.6	0.8195	1.2304	114	77.1
		No.2.	8	6	5	8	5					
		No.3.	10	9	8	6	9					
		No.4.	5	5	7	5	8					
		No.5.	6	6	5	5	8					
		No.6.	5	6	5	7	8					
		No.7.	9	5	5	6	5					
		No.8.	8	6	8	6	7					

TABLE 83

ANALYSIS OF VARIANCE OF COUNTS ON SAMPLES OF DIFFERENT DILUTIONS OF FILTERED SUSPENSION OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

(Bullock and Keepe's Method).

Dilution	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
Initial	(a) Between samples	240	4	60.0	1.81	0.1-0.2
	(b) Within samples	662	20	33.1		
	Total	902	24			
(18.3) fold	(a) Between samples	228	4	57.0	2.088	0.1-0.2
	(b) Within samples	546	20	27.3		
	Total	774	24			
$(18.3)^2$ fold	(a) Between samples	135	4	33.75	2.200	0.1-0.2
	(b) Within samples	307	20	15.35		
	Total	442	24			
$(18.3)^3$ fold	(a) Between samples	81	9	9.0	1.68	0.1-0.2
	(b) Within samples	107	20	5.35		
	Total	188	29			

TABLE 84

ANALYSIS OF VARIANCE OF COUNTS ON SAMPLES OF DIFFERENT DILUTIONS OF FILTERED SUSPENSION OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

(Centrifuging Method)

Dilution	Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P
Initial	(a) Between samples	155	3	51.66	2.175	0.1 - 0.2
	(b) Within samples	380	16	23.75		
	Total	535	19			
(18.3) fold	(a) Between samples	75	3	25.0	2.249	0.1 - 0.2
	(b) Within samples	179	16	11.2		
	Total	254	19			
$(18.3)^2$ fold	(a) Between samples	111	3	37.0	1.911	0.1 - 0.2
	(b) Within samples	310	16	19.37		
	Total	422	19			
$(18.3)^3$ fold	(a) Between samples	24	7	3.43	1.769	0.1 - 0.2
	(b) Within samples	62	32	1.94		
	Total	86	39			

Bullock and Keepe, whereas only about 76-83 per cent of the expected number was obtained by the centrifuging method. This result was unexpected because the centrifuging method had hitherto given a higher count than did the method of Bullock and Keepe.

The minimal size of aggregates sedimenting in 30 minutes through 1 millimetre of oil at 8300 g. was calculated from Stoke's law to be about 12 microns. Thus, all the aggregates of the organisms having dimensions greater than 12 microns sedimented through the oil during the 30 minutes centrifuging and passed into the Ringer's solution. The fact that the expected number of organisms was not recovered from the Ringer's solution suggested that about 17-24 per cent of the organisms were present as aggregates having dimensions less than 12 microns. About 97 per cent of the organisms in the unfiltered highly concentrated suspension were in aggregates having dimensions greater than 34 microns. These large aggregates sedimented rapidly and, during sedimentation, the smaller aggregates adhered to the larger and were thus carried down with them into the Ringer's solution. In the dilute filtered suspension the maximal size of the aggregates was only about 35 microns, and thus there were no large rapidly sedimenting aggregates to which small aggregates could adhere. Hence, many small aggregates still remained in the oil after 30 minutes centrifuging.

The time required for an individual spore to sediment through 1 millimetre of oil has been calculated to be about 60 minutes. Thus, if the suspension is centrifuged for that time, all the spores should sediment through the oil to the Ringer's solution. A filtered suspension containing a known number of organisms was centrifuged over sterile Ringer's solution for times up to 60 minutes (Table 85). About 71 per cent of the organisms were obtained in the Ringer's solution after 30 minutes centrifuging, about 86 per cent after 45 minutes and about 104 per cent of the expected number of organisms after centrifuging for 60 minutes. About 30 per cent of the organisms must, therefore, have been present in the oil as aggregates of less than 12 microns diameter while about 14 per cent were present as aggregates of less than 4 microns.

Thus it can be concluded that it is possible to transfer all the organisms originally in the oil into the Ringer's solution by the centrifuging method, provided the centrifuging time is sufficient to allow a single spore to sediment. This conclusion was confirmed by repeating the serial dilutions of the filtered suspension and performing viable counts on samples from each dilution by both Bullock and Keepe's method and the centrifuging method. The centrifuging time was standardised at 60 minutes. Once again a

TABLE 85

RELATIONSHIP BETWEEN TIME OF CENTRIFUGING INFECTED AND FILTERED LIGHT LIQUID PARAFFIN - RINGER'S SOLUTION SYSTEM AND VIABLE COUNT OF SPORES OF BACILLUS SUBTILIS IN RINGER'S SOLUTION

Expected number of organisms :- 571721/ml. of oil.

Time of Centrifuging	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
15 Minutes	No.1.	85	80	85	79	74	80.35	1.9050	3.6196	334,650	58.53
	No.2.	89	77	80	82	87					
	No.3.	84	73	79	73	78					
	No.4.	85	80	76	85	74					
30 Minutes	No.1.	100	90	104	99	95	98.8	1.9948	3.6196	411,500	71.8
	No.2.	103	100	90	99	103					
	No.3.	106	103	105	93	102					
	No.4.	104	97	103	90	90					

TABLE 85 Cont'd.

Time of Centri-fuging	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilu-tion Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms ob-tained
45 Minutes	No.1.	122	110	109	114	109	117.6	2.0705	3.6196	489,800	85.67
	No.2.	125	120	119	113	126					
	No.3.	120	127	120	112	116					
	No.4.	114	127	115	115	119					
60 Minutes	No.1.	141	147	157	142	140	142.2	2.1529	3.6196	592,405	103.62
	No.2.	138	136	140	145	133					
	No.3.	142	137	150	136	143					
	No.4.	143	149	134	135	140					

higher count was obtained by the centrifuging method than by the method of Bullock and Keepe (Tables 86-87).

It can be positively concluded that a suspension of organisms in oil consists of aggregates of varied sizes. It appears that no worker has yet succeeded in obtaining a suspension of discrete free living organisms in oil. Furthermore, previous workers do not appear to have realised that their suspensions contained large aggregates of organisms rather than single cells. The present work has underlined the real and great difficulties that exist in preparing a uniform suspension of individual cells in oils. Bullock and Booth (1953) claimed to have diluted a suspension of spores of Bacillus subtilis in oil, and obtained the expected number of organisms uniformly distributed in the dilute suspension. They used samples of oil which were ten times the volume of those used in the present work. The statistical analysis of the counts obtained for their dilute suspension shows that there was a significant difference between the number of viable organisms in their samples. They attributed this "slight significant difference" to extremely low counts of organisms in the oil, but, in the light of the present work, it appears that it might have been due to the presence of small aggregates of the spores.

The technique described above does, however, appear to produce more uniform suspensions than can have been employed by Bullock *et al* and can, therefore, be assumed to be an improvement on the simple method of triturating dried organisms with oil.

TABLE 86

DILUTION OF FILTERED SUSPENSION OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

(Method of Bullock and Keepe)

Dilution	Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
Initial		No. 1	100	94	96	100	90	97.68	1.9898	3.7661	571,037	100.00
		No. 2	94	104	90	90	94					
		No. 3	100	112	104	98	100					
		No. 4	96	102	106	92	93					
		No. 5	102	90	99	103	93					
(18.3) fold	31204	No. 1	32	41	39	36	39	41.72	1.6203	2.8675	30747	98.54
		No. 2	44	37	44	43	42					
		No. 3	46	40	42	46	39					
		No. 4	47	46	39	38	45					
		No. 5	42	42	49	42	43					

TABLE 86 Cont'd.

Dilution	Expected Number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
$(18.3)^2$ fold	1705	No.1.	92	88	82	90	91	89.00	1.9494	1.2788	1691	99.18
		No.2.	89	90	90	82	84					
		No.3.	89	92	95	89	90					
		No.4.	82	81	90	91	92					
		No.5.	89	92	96	86	93					
$(18.3)^3$ fold	93	No.1.	9	8	7			9.5	0.9777	0.9823	91	97.85
		No.2.	12	9	9							
		No.3.	8	8	9							
		No.4.	10	10	9							
		No.5.	10	11	10							
		No.6.	9	10	6							
		No.7.	10	8	7							
		No.8.	10	10	12							
		No.9.	9	12	9							
		No.10.	10	10	14							

TABLE 87

DILUTION OF FILTERED SUSPENSION OF SPORES OF BACILLUS SUBTILIS IN LIGHT LIQUID PARAFFIN.

(Centrifuge Method)

Time of centrifuging :- 60 minutes.

Dilution	Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
Initial	571,037	No.1.	125	138	129	136	134	137.40	2.1380	3.6195	572,133	100.19
		No.2.	138	126	136	140	139					
		No.3.	144	142	148	135	135					
		No.4.	142	143	147	135	136					
		No.5.										
(18.3) fold	31204	No.1.	111	119	107	109	113	107.60	2.0314	2.4624	31204	100.00
		No.2.	97	108	107	99	108					
		No.3.	109	104	101	111	100					
		No.4.	104	109	115	104	117					
		No.5.										

TABLE 87 Cont'd.

Dilution	Expected number of organisms per ml. of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
$(18.3)^2$ fold	1705	No.1.	50	45	50	48	41	50.1	1.6998	1.5315	1703	99.89
		No.2.	45	44	50	51	52					
		No.3.	48	56	50	58	50					
		No.4.	52	56	48	50	58					
		No.5.										
$(18.3)^3$ fold	93	No.1.	6	5	6	5	5	5.5	0.7404	1.2304	93	100.00
		No.2.	6	6	4	6	4					
		No.3.	5	4	4	6	6					
		No.4.	5	6	7	6	7					
		No.5.	5	5	7	8	7					
		No.6.	5	5	5	7	4					
		No.7.	5	6	7	5	6					
		No.8.	5	4	6	5	6					

(G) MIGRATION OF DRY "UNCOATED" BACTERIUM COLI FROM LIGHT LIQUID PARAFFIN TO RINGER'S SOLUTION.

Bacterium coli embedded in peptone particles were observed to sediment in light liquid paraffin very rapidly due to the abnormally large size of the particles. All the organisms initially present in the oily layer passed through to an underlying Ringer's solution within 30 minutes (Table 30). Dry "uncoated" spores of Bacillus subtilis in the same oil sedimented much more slowly, and the great majority of them collected at the interface between the oil and the Ringer's solution. It was concluded from the rate of sedimentation that the suspension of dry "uncoated" spores of Bacillus subtilis in the oil consisted of aggregates of organisms of varied sizes, and not of individual discrete cells. Similar experiments were performed to study the migration of dry "uncoated" Bacterium coli from oil to aqueous phase.

I. Sedimentation of Dry "Uncoated" Bacterium coli through Light Liquid Paraffin.

Fifteen millilitres of light liquid paraffin containing a known number of dry "uncoated" Bacterium coli were floated over 10 millilitres of sterile Ringer's solution in a series of separators. After allowing the two phases

to remain in contact for specified intervals of time, 0.52 millilitre samples of oil were carefully taken at each time interval from a separator at depths of 1,2 and 3 centimetres below the surface. The Ringer's solution was also collected from the same separator at each sampling time. Viable counts were performed on these samples and on a sample of the initial oily suspension which had been continuously rotated (Tables 88-92). Light liquid paraffin was highly bactericidal to dry "uncoated" Bacterium coli (Table 42) and, therefore, the sedimentation of the organisms in the oily phase could be studied only over a very short period. The viability of the organisms in the oil varied substantially from suspension to suspension. It was, therefore, determined simultaneously on the samples of oil kept rotating when the viable counts were performed on the samples of oil taken from different levels in the separators. When calculating the number of organisms in a sample of oil from a separator, allowance was made for the mortality in the oil during the sedimenting period, and for the mortality in petroleum ether.

The minimal size of aggregates that would sediment through 1,2 or 3 centimetres of oil during $1\frac{1}{2}$, 3, $4\frac{1}{2}$ and 6 hours has been calculated previously from Stoke's law and found to range from 416 to 120 microns. The maximal

TABLE 88

SEDIMENTATION OF DRY "UNCOATED" BACTERIUM COLI THROUGH 1 CENTIMETRE OF LIGHT LIQUID PARAFFIN.

Initial number of organisms in oil :- 493,073/ml.

Time of Sedimentation	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of viable organisms per ml. of oil	Percentage viability of organisms in initial suspension of oil	Total number of organisms per ml. of oil	Number of organisms sedimenting per ml of oil	Percentage of initial number of organisms sedimenting in oil.
1½ Hours	31	25	30	28	25	27.80	1.4440	3.3966	69277	17.21	402,540	90533	18.36
3 Hours	124	137	138	125	134	131.6	2.1192	2.1903	20382	5.90	345,460	148613	30.14
4½ Hours	102	116	113			110.3	2.0425	1.8915	8592	2.97	289,293	203,780	41.33
6 Hours	30	29	36			31.7	1.5011	1.8915	2469	1.04	237,410	255,663	51.85

TABLE 89

SEDIMENTATION OF DRY "UNCOATED" BACTERIUM COLI THROUGH 2 CENTIMETRES OF LIGHT LIQUID PARAFFIN.

Initial Number of organisms in oil:- 493,073/ml.

Time of Sedimentation	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of viable organisms per ml of oil	Percentage viability of organisms in initial suspension of oil	Total number of organisms per ml of oil	Number of organisms sedimenting per ml of oil	Percentage of initial number of organisms sedimenting in oil
1½ Hours	32	29	26	33	27	29.4	1.4683	3.3966	73264	17.21	425,706	67367	13.66
3 Hours	133	146	135	143	147	140.8	2.1485	2.1903	21824	5.90	369,899	123,174	24.98
4½ Hours	116	129	112			118.7	2.0745	1.8915	9246	2.97	311,314	181,759	36.86
6 Hours	30	38	36			34.7	1.5403	1.8915	2703	1.04	259,910	233,163	47.29

TABLE 90

SEDIMENTATION OF DRY "UNCOATED" BACTERIUM COLI THROUGH 3 CENTIMETRES OF LIGHT LIQUID PARAFFIN.

Initial number of organisms in oil :- 493,073/ml.

Time of Sedimentation	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of viable organisms per ml. of oil	Percentage viability of organisms in initial suspension of oil	Total number of organisms per ml. of oil	Number of organisms sedimenting per ml. of oil	Percentage of initial number of organisms sedimenting in oil
1½ Hours	35	27	33	27	27	29.8	1.4742	3.3966	74261	17.21	432,424	60649	12.30
3 Hours	149	164	165	151	151	156.0	2.1931	2.1903	24180	5.90	409,831	83242	16.88
4½ Hours	120	132	125			125.7	2.0993	1.8915	9784	2.97	329,428	163645	33.19
6 Hours	32	39	39			36.7	1.5647	1.8915	2858	1.04	274,810	218263	44.26

TABLE 91

MIGRATION OF BACTERIUM COLI FROM LIGHT LIQUID PARAFFIN INTO RINGER'S SOLUTION AND THEIR MULTIPLICATION IN THE AQUEOUS PHASE.

Total number of viable organisms in the oil :- 7,396,095.

Time of Contact between two phases	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of viable organisms in Ringer's solution	Number of viable organisms in Ringer's solution expressed as percentage of initial number of viable organisms in oil.
1½ Hours	38	46	45	37	44	42.0	1.6232	3.7378	229,656	3.10
3 Hours	74	88	87	77	87	82.6	1.9170	3.7378	451,656	6.10
4½ Hours	88	96	87	95	97	94.6	1.9759	3.7378	517,272	6.99
6 Hours	134	149	134	148	145	142.0	2.1523	3.7378	776,456	10.50
24 Hours	85	79	90	78	84	83.2	1.9201	4.9990	8,302,112	112.25
48 Hours	137	138	123	123	132	128.6	2.1093	5.8198	84,945,310	1148.52
96 Hours	120	135	119	133	127	126.8	2.1032	6.1173	166,139,950	2246.30

TABLE 92

SEDIMENTATION OF DRY "UNCOATED" BACTERIUM COLI THROUGH LIGHT LIQUID PARAFFIN, THEIR MIGRATION AND MULTIPLICATION INTO RINGER'S SOLUTION.

Sedimentation Time	Organisms Sedimenting Through						Mean % of initial number of organisms sedimenting through oil	% of initial number of viable organisms migrating into Ringer's solution and then multiplying in it.
	1 Centimetre		2 Centimetre		3 Centimetre			
	Theoretical minimal size of aggregates in microns	% of initial number of organisms	Theoretical minimal size of aggregates in microns	% of initial number of organisms	Theoretical minimal size of aggregates in microns	% of initial number of organisms		
1½ Hours	240	18.36	340	13.66	416	12.30	14.77	3.10
3 Hours	170	30.14	240	24.98	294	16.88	24.00	6.10
4½ Hours	155	41.33	196	36.86	240	33.19	37.13	6.99
6 Hours	120	51.85	170	47.29	208	44.26	47.8	10.55
24 Hours								112.25
48 Hours								1148.52

dimension of a single Bacterium coli is about 2.0 microns (Topley and Wilson - 1948 and Bergey - 1948) and the time required for such a particle to sediment through the column of oil (i.e., 4 centimetres) was calculated to be about 1.73×10^4 hours. After 1½, 3, 4½ and 6 hours the number of organisms which had sedimented through the oil was about 15, 24, 37 and 46 per cent respectively of the initial number of organisms in the oil. Thus it can be concluded from the rate of sedimentation that the sedimented organisms were in the oil in the form of aggregates whose minimal size ranged from 416 to 120 microns. In a sample of the initial suspension which was continuously rotated, there was about 1 per cent survivors after 6 hours and no survivors after 24 hours. It was, therefore, not possible to extend the sedimentation studies beyond six hours because after that time the small number of survivors made counting impracticable.

(a) Dilution of a suspension of dry "uncoated" Bacterium coli in light liquid paraffin.

Sedimentation experiments with dry "uncoated" Bacterium coli in light liquid paraffin showed that the suspension of organisms in the oil consisted, like the suspension of spores of Bacillus subtilis, of aggregates

of varied sizes. Equal volumes of this suspension contained equal numbers of viable organisms. The concentration of the organisms in the oil was high and thus viable counts again did not reveal the heterogeneity of the suspension produced by the varying sizes of aggregates. An attempt was made to reveal this heterogeneity by diluting the concentrated suspension.

Fifty drops of a suspension containing a known number of dry "uncoated" Bacterium coli in oil were added to 5.0 millilitres of sterile oil and the mixture shaken vigorously. No viable organisms could be detected in the dilute suspension, although a count of approximately half a million organisms per millilitre of oil was expected. The experiment was done in triplicate and each time five samples of the dilution were found to contain no viable organisms. It was inferred from this that the organisms died very rapidly in the dilute suspension, suggesting that a relationship existed between the concentration of the organisms in the oil and the mortality rate. The bactericidal action of substances in aqueous solutions is known to be greatly influenced by the concentration of the organisms (Rahn - 1945), but there is no information as to whether this relationship holds for an oily system. Experiments were, therefore, conducted to establish whether such a relationship did exist between inoculum size and mortality rate in an

oily system.

Ten drops of infected light liquid paraffin were added to each of two series of five sterile centrifuge tubes. To the tubes of each series were added 0,10,20,30 or 40 drops respectively of sterile light liquid paraffin and the contents mixed thoroughly. Viable counts were performed (a) on the contents of one series of five tubes immediately after preparation of the dilutions (Table 93), and (b) on the contents of the second series after allowing the dilutions to stand for 30 minutes (Table 94).

Into each tube was placed 10 drops of infected oil and, therefore, equal numbers of organisms. Each tube received exactly 3 millilitres of petroleum ether and thus the relationship between the number of viable organisms added to each tube and the volume of petroleum ether was constant.

A marked linear correlation ($r = 0.985$) was found to exist between the dilution of the original suspension and the mortality rate of the organisms (Fig.X). The rate of death of Bacterium coli on storage in light liquid paraffin was enhanced on dilution of the original suspension. In a suspension containing approximately $3 \cdot 5 \times 10^6$ organisms per millilitre of oil, about 35 per cent of the organisms

TABLE 93

DILUTION OF SUSPENSION OF DRY "UNCOATED" BACTERIUM COLI IN LIGHT LIQUID PARAFFIN.

(Counts performed immediately after dilution).

Dilution	Expected number of organisms per ml. of oil	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained
Initial		138	156	140	154	155	148.6	2.1721	4.3809	3,572,938	100.00
2 fold	1,786,469	92	81	90	80	91	86.8	1.9385	4.0727	1,024,218	57.33
3 fold	1,190,979	62	74	76	76	66	70.8	1.8500	3.6193	294,669	24.74
4 fold	893,234	50	61	63	53	51	55.6	1.7451	3.4945	173,583	19.41
5 fold	714,588	34	44	45	34	46	40.6	1.6085	3.3974	101,378	14.22

TABLE 94

DILUTION OF SUSPENSION OF DRY "UNCOATED" BACTERIUM COLI IN LIGHT LIQUID PARAFFIN.

(Counts performed after storing the dilutions for $\frac{1}{2}$-hour).

Dilution	Expected number of organisms originally present per ml of oil	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of oil obtained	Percentage of expected number of organisms obtained.
Initial	3,572,938	110	95	94	108	98	101.1	2.0043	4.0965	1,264,591	35.29
2 fold	1,786,469	40	52	39	50	47	45.6	1.6590	3.7944	346,276	19.38
3 fold	1,190,979	88	74	84	74	75	79.0	1.8976	2.7132	41001	3.44
4 fold	893,234	68	79	71			72.77	1.8619	1.9868	7058	0.79
5 fold	714,588	0	0	0			0.00	0.0000	1.8921	0	0.00

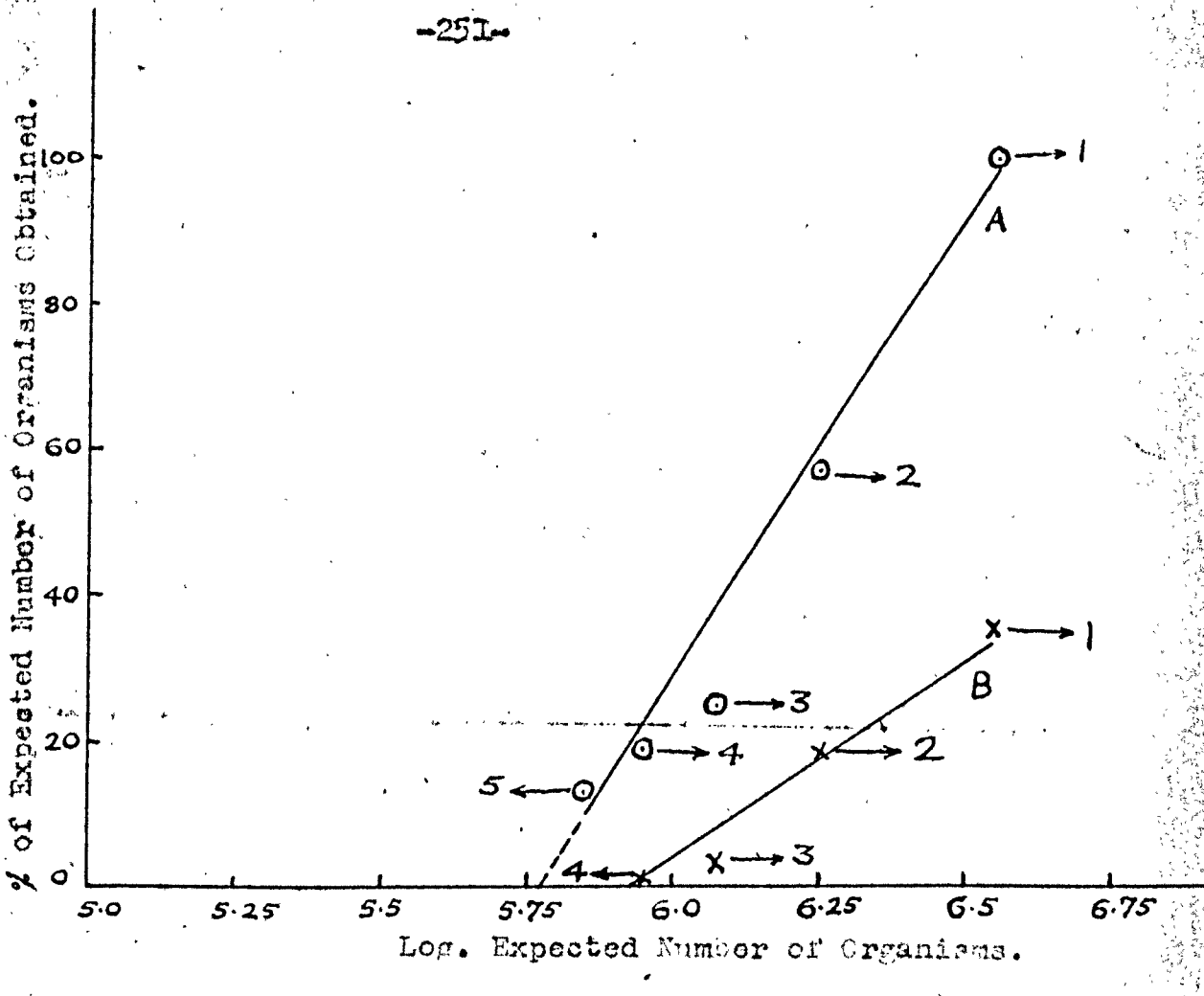


Fig. X Relationship between Dilution of a Suspension of Dry "Uncoated" Bacterium coli in Light Liquid Paraffin.

A :- Counts performed immediatly after dilution.
B :- Counts performed after ½-hour storage of dilutions.

(1) Initial suspension.
(2) Two-fold dilution.
(3) Three-fold dilution.
(4) Four-fold dilution.
(5) Five-fold dilution.

were still viable after storage for 30 minutes. In a suspension containing initially approximately 0.1 x 10^6 organisms per millilitre of oil, all the organisms died during the same period of storage. In fact, an extrapolation of the curve relating dilution of the suspension to immediate mortality (A) suggested that all the organisms were immediately killed in an approximately six-fold dilution of the original suspension. The absence of any viable organisms in the ten-fold dilution made in the initial experiments may, thus, be attributed to the almost extremely rapid death which occurred in the dilute suspension.

It can be concluded, that, contrary to the finding of Bullock and Keepe (1951), light liquid paraffin is highly bactericidal to dry vegative cells, and this bactericidal action is related to the concentration of the organisms in the oil. The rapid mortality of Bacterium coli in the oil precluded the possibility of employing a diluted suspension for further studies.

(b) The enumeration of dry "uncoated" Bacterium coli in light liquid paraffin by the centrifuging method.

It has been shown (Page 204) that an estimate of the number of viable spores of Bacillus subtilis in an oil can be obtained by floating a sample of the infected oil

over Ringer's solution, centrifuging the system at 8300 g., and performing a viable count on the Ringer's solution. Since the method proved satisfactory for enumerating spores in oil, an attempt was made to apply the technique to a sample of oil infected with dry "uncoated" Bacterium coli.

A viable count was performed on the contents of a tube containing freeze-dried "uncoated" Bacterium coli. The counts of viable organisms in the tube can be regarded as representative of the counts in any of the remaining tubes of the same batch, since it has been shown that there is no significant difference in the number of viable organisms in the freeze-dried tubes from the same batch (Table 35) The contents of a second tube from the same batch were added to 25 millilitres of light liquid paraffin, and shaken vigorously to produce a concentrated heterogeneous suspension. Viable counts were performed on 0.52 millilitre samples of the infected oil by both the method of Bullock and Keepe and the centrifuging method, the centrifuging time being 30 minutes at 8300 g. (Tables 95-96).

The viable counts performed by the method of Bullock and Keepe detected 100 per cent of the organisms used to infect the oil (after allowing for the mortality in petroleum ether), while the viable counts performed by the centri-

TABLE 95

ENUMERATION OF VIABLE BACTERIUM COLI IN LIGHT LIQUID PARAFFIN BY BOTH METHODS.

Experiment		Expected number of organisms per ml of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml of oil obtained	Percentage of expected number of organisms obtained.
I	Bullock and Keepe's Method	19,924,824	No.1.	218	210	224	227	221	213.88	2.3385	4.9696	19,942,600	100.09
			No.2.	210	202	206	219	200					
			No.3.	209	221	216	214	211					
			No.4.	202	216	212	218	208					
			No.5.	224	208	210	218	223					
	Centrifuging Method	19,924,824	No.1.	50	62	52	53	57	53.0	1.7243	5.1093	6,818,100	34.22
			No.2.	55	54	54	65	52					
			No.3.	55	47	45	52	52					
			No.4.	54	48	58	49	46					

TABLE 95 Cont'd.

Experiment		Expected number of organisms per ml of oil	Sample	Counts of 5-replicate tubes					Mean Count	Log Mean Count	Log. Dilution Factor	Number of organisms per ml of oil obtained	Percentage of expected number of organisms obtained
II	Bullock and Keepe's Method	15,878,720	No.1.	82	78	94	86	92	84.36	1.9261	5.2704	15,731,790	99.08
			No.2.	84	84	86	94	89					
			No.3.	80	88	83	70	81					
			No.4.	80	89	91	90	84					
			No.5.	78	84	76	86	80					
	Centrifuging Method	15,878,720	No.1.	60	54	58	65	55	54.0	1.7324	5.1093	6,946,700	43.75
			No.2.	62	50	53	57	53					
			No.3.	53	48	58	49	48					
			No.4.	55	49	45	53	55					

TABLE 96

ANALYSIS OF VARIANCE OF COUNTS OF BACTERIUM COLI IN LIGHT LIQUID PARAFFIN BY BOTH METHODS.

Experiment		Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio	P.
I	Bullock and Keepe's Method	(a) Between samples	471	4	117.75	2.726	0.05 - 0.1
		(b) Within samples	864	20	43.2		
		Total	1335	24			
	Centrifuging Method	(a) Between samples	120	3	40.0	1.778	0.1 - 0.2
		(b) Within samples	360	16	22.5		
		Total	480	19			
II	Bullock and Keepe's Method	(a) Between samples	239	4	59.75	1.995	0.1 - 0.2
		(b) Within samples	579	20	28.95		
		Total	818	24			
	Centrifuging Method	(a) Between samples	175	3	58.33	2.962	0.05 - 0.1
		(b) Within samples	313	16	19.56		
		Total	488	19			

fuging method detected only about 40 per cent of the initial inoculum. The low recovery obtained by the latter method indicated that many organisms were being lost during the centrifuging process.

It was observed that during centrifuging, the temperature of the solutions in the centrifuge rose to about 43° - 45°C, probably due to air-friction. It was thought probable that the rise in temperature was responsible for some of the organisms dying during this period of centrifuging. To ascertain whether or not the rise in temperature was responsible for the death of organisms, comparative experiments were performed in which Bacterium coli were suspended in Ringer's solution at 45°C and room temperature.. Tables 97 and 98 record the survival of Bacterium coli in Ringer's solution at these temperatures. After 30 minutes storage at 45°C only about 35 per cent of the organisms were still viable, while at room temperature there was nil mortality in the same period. Thus, it appeared that the rise in temperature during centrifuging was responsible for the low counts of Bacterium coli.

To confirm that the loss of organisms during centrifuging was due to the progressive rise of temperature in the centrifuge, the oily suspension - Ringer's solution system was centrifuged for 5,10,20 and 30 minutes respectively and viable counts performed on the Ringer's solutions (Table 99). After centrifuging for 5 minutes, 100 per cent

TABLE 97

THE SURVIVAL OF BACTERIUM COLI IN QUARTER-STRENGTH RINGER'S SOLUTION AT 45°C.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of suspension	Percentage Survival
0 Hour	198	202	193	207	204	200.2	2.3012	3.8560	1,437,235	
½ Hour	69	72	62	75	70	69.6	1.8426	3.8560	499,658	34.77
1 Hour	51	46	58	58	46	51.8	1.7143	2.8585	37399	2.60
2 Hours	80	71	70	66	79	73.2	1.8645	1.9823	7027	0.48
4 Hours	46	56	44	57	58	52.2	1.7177	1.9823	5011	0.34

TABLE 98.

THE SURVIVAL OF BACTERIUM COLI IN QUARTER-STRENGTH RINGER'S SOLUTION AT ROOM TEMPERATURE.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of suspension	Percentage Survival
0 Hour	204	189	192	203	193	196.2	2.2927	3.8560	1,408,519	
½ Hour	190	204	203	206	192	199.0	2.2989	3.8560	1,428,621	101.43
1 Hour	189	204	201	191	202	197.4	2.2954	3.8560	1,417,134	100.61
2 Hours	186	199	201	187	199	194.4	2.2887	3.8560	1,395,597	99.08
4 Hours	191	204	205	203	192	199.0	2.2989	3.8560	1,428,621	101.43

TABLE 99

RELATIONSHIP BETWEEN TIME OF CENTRIFUGING INFECTED LIGHT LIQUID PARAFFIN-RINGER'S SOLUTION SYSTEM AND VIABLE COUNT OF BACTERIUM COLI IN RINGER'S SOLUTION.

Expected Number of Organisms :- 875,170/ml. of oil.

Time of Centrifuging	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of viable organisms per ml. of oil obtained	Percentage of expected number of organisms obtained in viable state
5 Minutes	No.1.	77	89	90	77	90	84.10	1.9248	4.0216	884,480	101.07
	No.2.	92	78	80	90	90					
	No.3.	72	82	86	74	80					
	No.4.	90	79	82	90	92					
10 Minutes	No.1.	75	62	74	62	74	73.4	1.8657	4.0216	771,950	88.21
	No.2.	78	70	80	69	71					
	No.3.	78	70	81	70	79					
	No.4.	74	69	81	71	70					

TABLE 99 Cont'd.

Time of Centrifuging	Sample	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of viable organisms per ml. of oil obtained	Percentage of expected number of organisms obtained in viable state
20 Minutes	No.1.	108	118	105	105	116	113.45	2.0550	3.6199	472,560	54.00
	No.2.	118	124	113	125	110					
	No.3.	105	116	104	106	119					
	No.4.	110	120	109	118	120					
30 Minutes	No.1.	78	65	78	65	77	76.1	1.8814	3.6199	316,956	36.22
	No.2.	81	73	84	72	74					
	No.3.	82	71	84	72	83					
	No.4.	74	82	80	70	77					

of the organisms used to infect the oil were obtained in the Ringer's solution in the viable state. The proportion of viable organisms obtained in the Ringer's solution after centrifuging for 10,20 and 30 minutes was 88,54 and 36 per cent respectively. This showed that the loss of viable organisms in the system was progressive. Thus, it can be deduced that the progressive rise of temperature in the centrifuge was responsible for the loss of organisms during centrifuging. It was not possible to control the temperature of the centrifuge used, but the results suggested that if the temperature of the centrifuge could be controlled, as can be done by using a refrigerated centrifuge, the method would be applicable to Bacterium coli.

II. Migration of Dry "Uncoated" Bacterium coli from Light Liquid Paraffin to Ringer's Solution.

The experiments on the migration of dry "uncoated" spores of Bacillus subtilis from an oil to an underlying aqueous phase revealed, that the majority of the spores that sedimented through the oil were adsorbed at the interface and did not pass to the aqueous phase. A similar adsorption of organisms at the interface was observed when spores of Bacillus subtilis were replaced by dry "uncoated" Bacterium coli. The total number of Bacterium coli that sedimented through

the oil during 6 hours was about 46 per cent of those initially in the oil, but only about 10 per cent penetrated through the interface into the Ringer's solution (Tables 91 - 92).

Viable counts performed on the Ringer's solution collected from a separator after 48 Hours contact between the two phases, showed that there were about 10 times as many viable organisms in the Ringer's solution as were present initially in the oil. This result was completely unexpected. However, further identical experiments on the migration of the organisms from oily to aqueous phase, produced similar results (Table 100). This unexpectedly high viable count was obtained from a system, the components of which were known to be bactericidal. About 98 per cent of organisms used to infect the oil had been killed during freeze-drying and about 99.0 per cent of the remaining viable organisms introduced into the oil were killed within 6 hours, and all were killed within 24 hours (Tables 34 and 42). About 1.0 x 10^6 viable organisms were found in the Ringer's solution after 6 hours contact with the infected oil, about 8 - 10 x 10^6 viable organisms after 24 hours contact, and about 60 - 70 x 10^6 viable organisms after 48 hours contact (Fig.XI). This increase in the count in the Ringer's solution cannot be attributed to the continual migration of viable organisms over

TABLE 100

MIGRATION OF BACTERIUM COLI FROM LIGHT LIQUID PARAFFIN INTO RINGER'S SOLUTION AND THEIR MULTIPLICATION IN THE AQUEOUS PHASE.

Total number of viable organisms in oil :- 3,226,230.

Experiment	Time of Contact between two phases	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of viable organisms in Ringer's solution	Number of viable organisms in Ringer's solution expressed as percentage of initial number of viable organisms in oil.
I	1½ Hours	140	153	139	138	150	144.0	2.1584	2.8797	108,600	3.36
	3 Hours	102	89	93	102	99	97.0	1.9868	3.1577	139,500	4.32
	4½ Hours	186	173	180	170	182	178.2	2.2509	3.1577	256,250	7.94
	6 Hours	261	280	266	276	265	269.6	2.4308	3.1577	387,700	12.02
	24 Hours	130	146	148	133	147	140.8	2.1485	4.4368	3,849,200	119.30
	48 Hours	119	118	104	118	106	113.0	2.0531	5.5120	36,738,200	1138.73
	96 Hours	230	243	247	229	244	238.6	2.3777	5.5120	76,727,600	2378.24

TABLE 100 Cont'd.

Total number of viable organisms in oil :- 3,768,345.

Experiment	Time of Contact between two phases	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of viable organisms in Ringer's solution	Number of viable organisms in Ringer's solution expressed as percentage of initial number of viable organisms in oil.
II	1½ Hours	77	81	90	79	90	83.4	1.9212	3.1577	119,930	3.18
	3 Hours	139	129	141	140	130	134.8	2.1297	3.1577	193,800	5.14
	4½ Hours	170	176	161	177	163	169.4	2.2290	3.1577	243,600	6.46
	6 Hours	270	286	291	286	273	281.2	2.4490	3.1577	404,360	10.73
	24 Hours	131	145	149	132	141	139.6	2.1449	4.4368	3,816,384	101.27
	48 Hours	114	120	130	127	119	122.0	2.0864	5.5120	39,664,000	1052.56
	96 Hours	203	200	217	218	207	209.0	2.3201	5.5120	67,945,000	1803.05

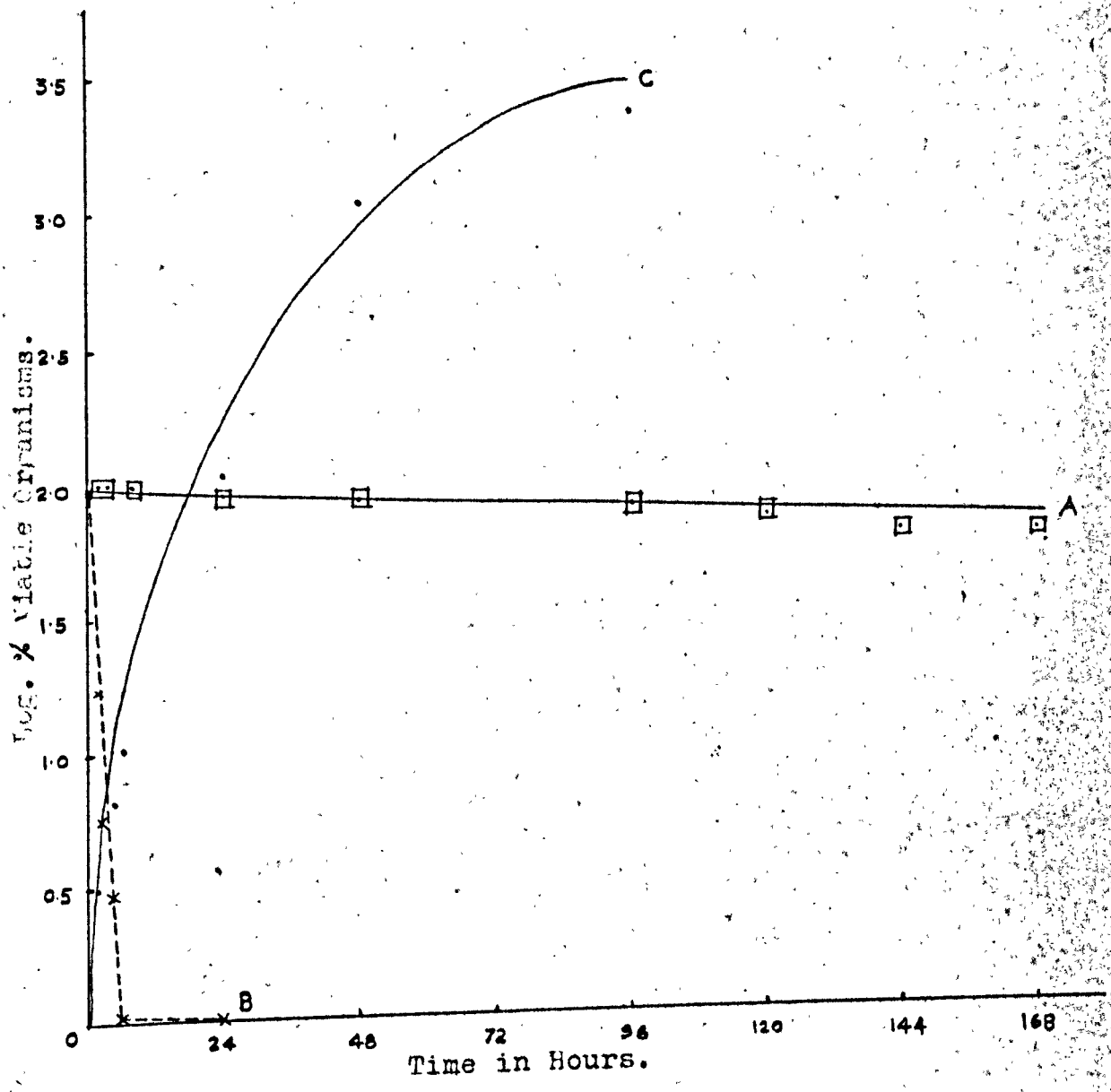


Fig.XI The Behaviour of Bacterium coli in Single and Two-Phase Systems.

A:- Ringer's solution.
B:- Light liquid paraffin.
C:- Light liquid paraffin-Ringer's solution system.

this period from the oil into the Ringer's solution. The increase in the count in the Ringer's solution observed between 6 and 48 hours contact with the oil must, therefore, be a real increase in the number of viable organisms present in the system. That is, multiplication of the organisms must have occurred in the system. This multiplication cannot have occurred in the oil, since the latter has been shown to be highly bactericidal to the organisms, and since water is essential for the synthesis of cellular contents (Fabian and Pivnick - 1953). It can only have occurred either in the Ringer's solution or at the interface between the oil and the Ringer's solution.

The two phases under examination have been shown to be bactericidal when separate; the organisms die extremely rapidly in the oil and slowly in the Ringer's solution. However, when the two phases are brought into contact, there is an initial reduction in the number of viable organisms in the system which is followed by a remarkable increase in the number of viable organisms.

Bacterial multiplication can only occur in a system containing sufficient nutrients to supply the metabolic needs of the organisms. Thus, the metabolic requirements of the organisms must have been fulfilled by a component of the system itself. An investigation was, therefore, undertaken to attempt to locate the origin and nature of the nutrients

that would permit a multiplication of Bacterium coli in light liquid paraffin - Ringer's solution system.

(H) DETERMINATION OF THE SITE OF MULTIPLICATION OF BACTERIUM COLI IN THE SYSTEM LIGHT LIQUID PARAFFIN - RINGER'S SOLUTION.

In order to elucidate the cause of multiplication of Bacterium coli observed when an infected oily phase was brought into contact with sterile Ringer's solution, it was first necessary to determine where the multiplication occurred. The possible sites of multiplication have been concluded to be either at the interface or in the aqueous phase.

To eliminate the possibility of the multiplication occurring at the interface where large numbers of organisms accumulated, the Ringer's solution was aseptically run off from the separator after 24 hours contact with the infected oil. Viable counts were performed on the separated Ringer's solution immediately after separation, and during storage up to 7 days. Parallel experiments were set up in which the Ringer's solution was separated after contact with the infected oil for 48, 96, 120, 144 and 168 hours, and viable counts performed immediately after separation (Tables 101-102).

Most of the organisms originally introduced into the oil had collected at the interface during 24 hours sedimentation, and a small proportion of them had penetrated into the

TABLE 101

MULTIPLICATION OF BACTERIUM COLI IN RINGER'S SOLUTION COLLECTED AFTER 24 HOURS CONTACT WITH LIGHT LIQUID PARAFFIN INFECTED WITH DRY "UNCOATED" BACTERIUM COLI

Initial number of viable organisms in oil :- 12,630,000.

Duration of Storage after contact	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Viable number of organisms in Ringer's solution
0 Hours	110	117	108	115	118	113.6	2.0554	5.0319	12,223,300
24 Hours	98	107	98	100	106	101.8	2.0076	6.1193	133,383,600
72 Hours	67	76	76	67	70	71.2	1.8525	6.5159	233,551,370
96 Hours	50	57	59	51	53	54.0	1.7324	6.5159	177,131,610
120 Hours	80	87	81	89	83	84.0	1.9243	6.1173	110,061,084
144 Hours	69	70	61	68	62	65.0	1.8129	6.1173	85,150,000

TABLE 102

MULTIPLICATION OF BACTERIUM COLI IN INFECTED LIGHT LIQUID PARAFFIN - RINGER'S SOLUTION SYSTEM.

Initial number of viable organisms in oil :- 12,630,000.

Duration of contact between two phases	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Total number of organisms in the Ringer's solution
24 Hours	110	117	108	115	118	113.6	2.0554	5.0319	12,223,300
48 Hours	107	100	109	101	110	105.4	2.0229	6.1173	138,100,600
96 Hours	70	80	78	71	79	75.6	1.8785	6.5159	247,984,320
120 Hours	52	58	61	52	55	55.2	1.7419	6.5159	181,067,868
144 Hours	80	81	84	88	82	83.0	1.9191	6.1173	108,750,833
168 Hours	62	69	59	68	63	64.2	1.8075	6.1173	84,102,000

Ringer's solution. After 24 hours contact between the two phases a viable count performed on the Ringer's solution revealed the presence of approximately as many viable organisms as were originally introduced into the oil. This count must be taken as an indication that some multiplication had already occurred, since only very few viable organisms have been found to penetrate from the oil into the Ringer's solution within 6 hours. During this time, the majority of organisms in the oil were killed (Tables 88-92).

The number of viable organisms in the Ringer's solution after 96 hours represented approximately 20-fold increase over the number originally viable in the oil. This 20-fold increase was observed in both the Ringer's solution that was separated after 96 hours contact with the infected oil, and in the Ringer's solution collected after 24 hours contact and subsequently stored for a further 72 hours. The multiplication, thus, clearly occurred within the bulk of the Ringer's solution and not at the interface. This conclusion must be accepted, even though Bacterium coli are known to die slowly when introduced into sterile Ringer's solution that has not been in contact with an infected oil.

(I) DETERMINATION OF THE SOURCE OF NUTRITIVE MATERIAL RESPONSIBLE FOR THE MULTIPLICATION OF BACTERIUM COLI IN THE SYSTEM LIGHT LIQUID PARAFFIN-RINGER'S SOLUTION.

Bacterium coli introduced into Ringer's solution that has not been in contact with an infected oil gradually die (Table 29), whereas Bacterium coli sedimenting from an oil into a volume of Ringer's solution in contact with the oil exhibit multiplication. Since Ringer's solution itself supplies no nutritive material, the latter must have been transferred to the Ringer's solution from the infected oil. Lee and Chandler (1941) have shown that organisms can multiply in soluble-oil emulsions prepared from mineral oils, and suggested that the naphthanic acids present in the oils can supply the nutritive materials.

To investigate whether light liquid paraffin could yield nutritive water-soluble materials, a volume of sterile light liquid paraffin was vigorously shaken with sterile Ringer's solution and the latter separated off. The separated Ringer's solution was inoculated with Bacterium coli and viable counts performed at intervals during seven days (Table 103). The viable count gradually decreased in a manner similar to that observed in Ringer's solution which had not been shaken with oil (Table 29). Thus, no

TABLE 103

THE SURVIVAL OF BACTERIUM COLI IN RINGER'S SOLUTION AFTER SHAKING WITH STERILE LIGHT LIQUID PARAFFIN.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of Ringer's Solution	Percentage Survival
0 Hour	133	125	129	125	136	129.6	2.1127	2.8690	93548	100.00
24 Hours	133	123	130	123	133	128.4	2.1086	2.8690	92682	99.08
48 Hours	131	122	131	122	134	128.0	2.1072	2.8690	92393	98.77
96 Hours	115	109	108	119	109	112.0	2.0492	2.8690	82835	88.55
120 Hours	104	101	100	110	103	104.6	2.0195	2.8690	77362	82.70
144 Hours	81	89	90	83	87	86.0	1.9345	2.8690	63605	67.99
168 Hours	70	78	69	79	76	74.4	1.8716	2.8690	55026	58.82

nutritive material was extracted from the oil by the Ringer's solution which could possibly account for the multiplication of the organisms observed when the two phases were in contact.

The other probable source of nutritive material was the organisms themselves. The possibility of organisms in water multiplying at the expense of dead cells has been suggested by Ficker (1898) and by Boissvain and Webb (1928).

Hotchkiss (1946), Gale and Taylor (1947), Salton (1951) and Bean and Walters (1955) have demonstrated that when bacteria die in dilute solutions of a bactericide, there is a leakage of water-soluble cellular constituents. Bean and Walters have further shown that these cell eluates can be utilised as nutritive materials by the last survivors in a bactericidal system, with a consequent increase in the viable count of the system. It is suggested that a similar mechanism was operating in the oil-Ringer's solution system. The dry inoculum introduced in the oil contained about 2 per cent of viable organisms and 98 per cent dead cells. A large proportion of the viable organisms introduced into the oil died during their passage through the oil. It is postulated that, either the cells at death released water-soluble constituents into the oil by virtue of their cell-walls being disrupted or rendered permeable, or that dead cells sedimented through the oil to the interface where

soluble cellular constituents,were extracted by the aqueous phase. Both mechanisms would produce in the Ringer's solution an eventual concentration of soluble cellular constituents of the type demonstrated by Bean and Walters (1955).

A viable count was performed on a suspension of dry "uncoated" Bacterium coli in light liquid paraffin. The suspension was stored for 48 hours to permit the inoculum to be killed by the oil. Fifteen millilitres of this stored suspension were then shaken vigorously with 10 millilitres of sterile Ringer's solution. The mixture was centrifuged at 8300 g. for 10 minutes to transfer all the organisms to the aqueous phase. The oil was pipetted off and the Ringer's solution was filtered through a bacteria-proof sintered glass filter to remove the bodies of the dead organisms. A further portion of Ringer's solution was shaken vigorously with sterile oil and used as a control. Both the control Ringer's solution and that shaken with the infected oil were inoculated with a suspension of Bacterium coli and viable counts performed immediately after inoculation, and at specified intervals during storage for 7 days (Tables 103, 104 and Fig. XII).

In the control Ringer's solution there was no multiplication but a gradual death of the organisms, the rate of which was similar to that observed for Bacterium coli

TABLE 1C4

MULTIPLICATION OF BACTERIUM COLI IN ELUATE EXTRACTED FROM LIGHT LIQUID PARAFFIN INFECTED WITH DRY "UNCOATED" BACTERIUM COLI.

Initial number of viable Organisms in the Oil :-14,360,000

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of Ringer's solution
0 Hour	118	116	126	127	123	122.0	2.0864	2.8690	88062
24 Hours	90	98	96	89	99	94.4	1.9750	3.8560	677,778
48 Hours	51	59	59	52	53	54.8	1.7388	5.3397	11,983,722
96 Hours	93	103	102	94	104	99.2	1.9965	5.9380	86,006,638
120 Hours	71	80	81	73	80	77.0	1.8865	5.9380	66,759,154
144 Hours	62	71	72	64	69	67.2	1.8274	5.6373	29,155,459
168 Hours	82	88	78	86	77	82.2	1.9149	5.3397	17,975,660

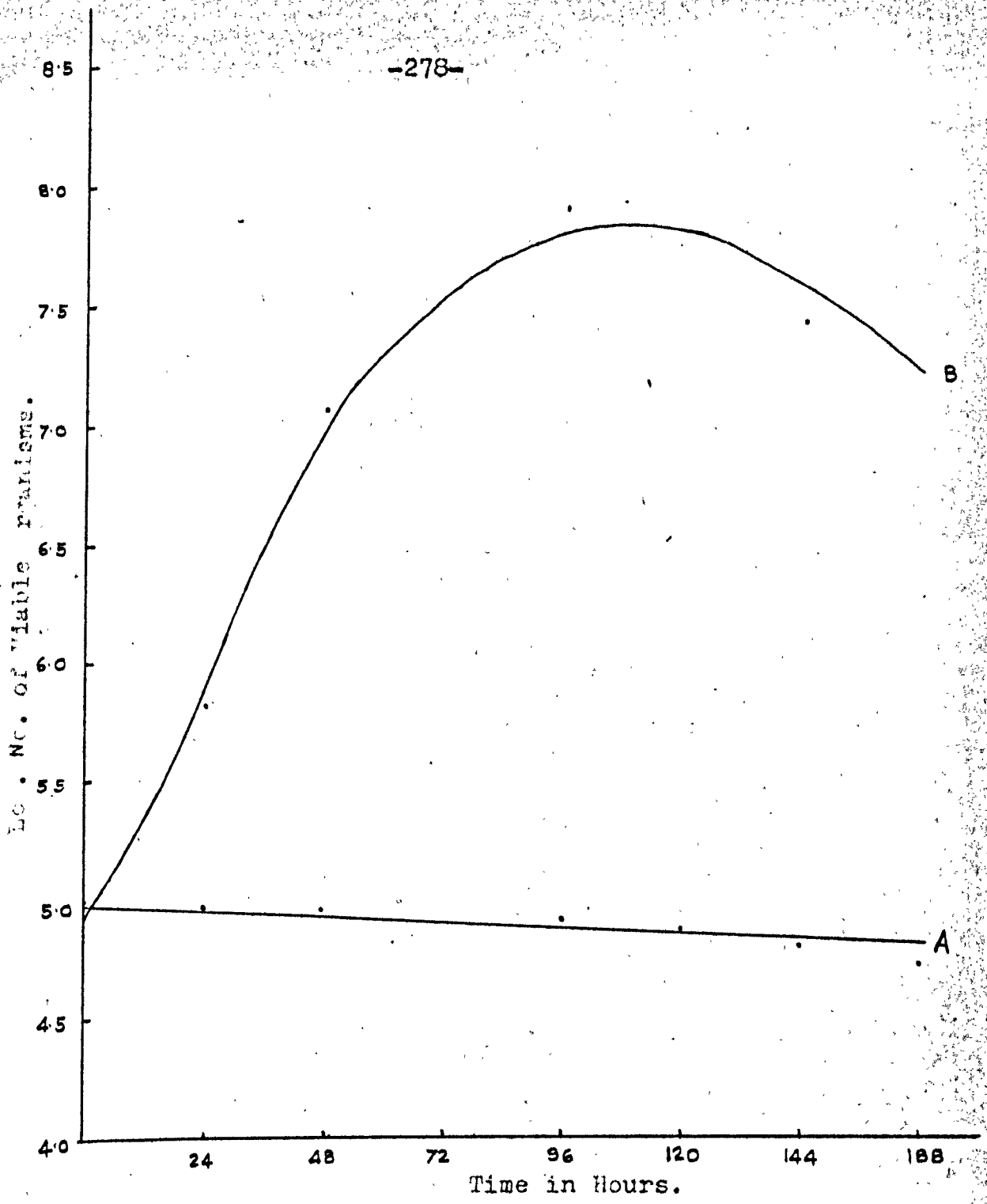


Fig. XII Multiplication of Bacterium coli in Aqueous Extracts of Light Liquid Paraffin.

A:- Viable counts in aqueous extract of sterile light liquid paraffin.

B:- Viable counts in aqueous extract of infected light liquid paraffin.

in Ringer's solution (Table 29).

A thousand-fold increase in the number of viable organisms was observed in 96 hours in the Ringer's solution that had been shaken with the infected oil and subsequently inoculated. After this period the organisms gradually died. This multiplication must have been due to some nutritive material which is not normally present in Ringer's solution and is not extractable by Ringer's solution from sterile light liquid paraffin. It must have been provided by the bacterial cells inoculated into the oil. Since the dead bacterial cells were removed by filtration after the oil and the Ringer's solution had been shaken together, the nutrient material must have been a soluble eluate from the dead cells.

Initially approximately 14 x 10^6 freeze-dried viable organisms were introduced into the oil (Table 104). Since there is a mortality of about 98 per cent during freeze-drying, the total number of viable and dead organisms introduced into the oil was 700 x 10^6, all of which must have contributed soluble cellular constituents to the Ringer's solution. These soluble cellular constituents extracted by the Ringer's solution were sufficient to produce a final crop in the Ringer's solution of about 86 x 10^6 organisms, that is, one-eighth of the total number of organisms originally inoculated into the oil.

The conclusion to be drawn from these observations is that if Bacterium coli, or possibly other vegetative organisms, are introduced into an oil, they will quickly die if the system is anhydrous. If, however, there is an adjacent aqueous phase, there may be in the aqueous phase an appreciable final crop of organisms which may persist for a period.

(J) THE RELATIONSHIP BETWEEN THE CONCENTRATION OF ELUATE FROM BACTERIUM COLI AND THE GROWTH RATE IN THE AQUEOUS PHASE

The quantity of eluate from organisms on death in oil would depend upon the total number of organisms initially introduced into the oil. If eluate was the sole source of nutrition, the rate of growth of bacteria added to the aqueous phase would probably depend on the concentration of eluate passing into the aqueous phase and consequently upon the total number of organisms initially present in the oil.

Five different samples of oil containing different concentrations of Bacterium coli were stored for 48 hours to allow the organisms to die. Fifteen millilitres of each of these suspensions were shaken with 10 millilitres portions of sterile Ringer's solution, the mixtures centrifuged for ten minutes and the Ringer's solutions filtered through a bacteria-proof sintered glass filter. The filtered Ringer's solutions were inoculated with a suspension of Bacterium coli and viable counts performed (Tables 105-109 and Fig. XIII).

The rate of multiplication of the organisms increased with the increase in the total number of organisms initially introduced into the oil and consequently with the increase in the amount of eluate obtained from them into the aqueous phase. When the oil was inoculated with

TABLE 108

MULTIPLICATION OF BACTERIUM COLI IN ELUATE EXTRACTED FROM LIGHT LIQUID PARAFFIN INFECTED WITH DRY "UNCOATED" BACTERIUM COLI.

Initial number of viable Organisms in oil :- 15,578,265.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of Organisms per ml. of Ringer's solution
0 Hour	265	248	264	250	266	258.6	2.4126	2.8585	286,663
24 Hours	90	99	89	98	89	93.0	1.9685	4.9412	8,123,550
48 Hours	64	74	69	74	68	69.8	1.8439	5.9380	60,516,739
96 Hours	109	103	113	102	111	107.6	2.0319	5.9380	93,289,415
120 Hours	74	80	71	70	75	74.0	1.8692	5.9380	64,158,148
144 Hours	93	86	83	92	82	87.2	1.9405	5.6373	37,832,592
168 Hours	60	53	59	60	50	56.4	1.7513	5.6373	24,469,704

TABLE 106

MULTIPLICATION OF BACTERIUM COLI IN ELUATE EXTRACTED FROM LIGHT LIQUID PARAFFIN INFECTED WITH DRY "UNCOATED" BACTERIUM COLI.

Initial number of viable Organisms in Oil :- 12,462,612.

Duration of storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of Ringer's solution
0 Hour	270	255	254	271	262	262.4	2.4190	2.8585	189,406
24 Hours	279	262	258	278	268	269.0	2.4298	3.8561	1,931,381
48 Hours	83	74	83	77	83	80.0	1.9031	4.9412	6,988,009
96 Hours	50	48	56	57	50	52.2	1.7177	5.3397	11,415,148
120 Hours	102	112	101	109	101	105.0	2.0212	4.6438	4,623,675
144 Hours	140	132	144	136	133	137.0	2.1367	4.3461	3,040,578
168 Hours	102	96	104	97	93	98.4	1.9930	4.3461	2,183,896

TABLE 107

MULTIPLICATION OF BACTERIUM COLI IN ELUATE EXTRACTED FROM LIGHT LIQUID PARAFFIN INFECTED WITH DRY "UNCOATED" BACTERIUM COLI.

Initial number of viable Organisms in Oil :- 9,346,959.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of Ringer's solution
0 Hour	274	282	266	286	266	274.8	2.4391	2.8585	198,356
24 Hours	92	82	88	89	93	88.8	1.9484	3.5551	318,785
48 Hours	112	106	106	102	113	107.8	2.0327	3.8561	773,988
96 Hours	144	136	135	147	142	140.8	2.1485	3.8561	1,010,944
120 Hours	92	94	100	90	92	93.6	1.9713	3.8561	672,048
144 Hours	87	84	86	85	80	84.4	1.9263	3.8561	577,272
168 Hours	70	77	68	75	68	71.6	1.8549	3.5551	257,044

TABLE 108

MULTIPLICATION OF BACTERIUM COLI IN ELUATE EXTRACTED FROM LIGHT LIQUID PARAFFIN INFECTED WITH DRY "UNCOATED" BACTERIUM COLI.

Initial number of viable organisms in oil :- 6,231,306.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of Organisms per ml. of Ringer's Solution
0 Hours	280	294	299	282	295	290.0	2.4624	2.8585	209,328
24 Hours	72	66	61	72	73	68.8	1.8376	3.5551	246,987
48 Hours	81	72	83	80	75	78.2	1.8932	3.5551	280,732
96 Hours	104	102	93	93	99	98.2	1.9921	3.5551	352,538
120 Hours	80	71	82	82	75	78.0	1.8921	3.5551	280,020
144 Hours	66	60	70	64	69	65.8	1.8182	3.5551	236,222
168 Hours	57	59	54	63	60	58.6	1.9129	3.5551	210,374

TABLE 109

MULTIPLICATION OF BACTERIUM COLI IN ELUATE EXTRACTED FROM LIGHT LIQUID PARAFFIN INFECTED WITH DRY "UNCOATED" BACTERIUM COLI.

Initial number of viable organisms in oil :- 3,115,653.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of Ringer's solution
0 Hour	262	258	270	277	275	268.4	2.4288	2.8585	193,784
24 Hours	99	110	107	106	109	106.2	2.0261	3.2608	193,602
48 Hours	95	105	106	98	106	102.0	2.0086	3.2608	185,946
96 Hours	92	103	92	96	100	96.6	1.9850	3.2608	176,101
120 Hours	90	99	89	95	98	94.2	1.9741	3.2608	171,726
144 Hours	88	89	97	92	98	92.4	1.9657	3.2608	168,445
168 Hours	84	93	83	92	88	88.0	1.9445	3.2608	160,424

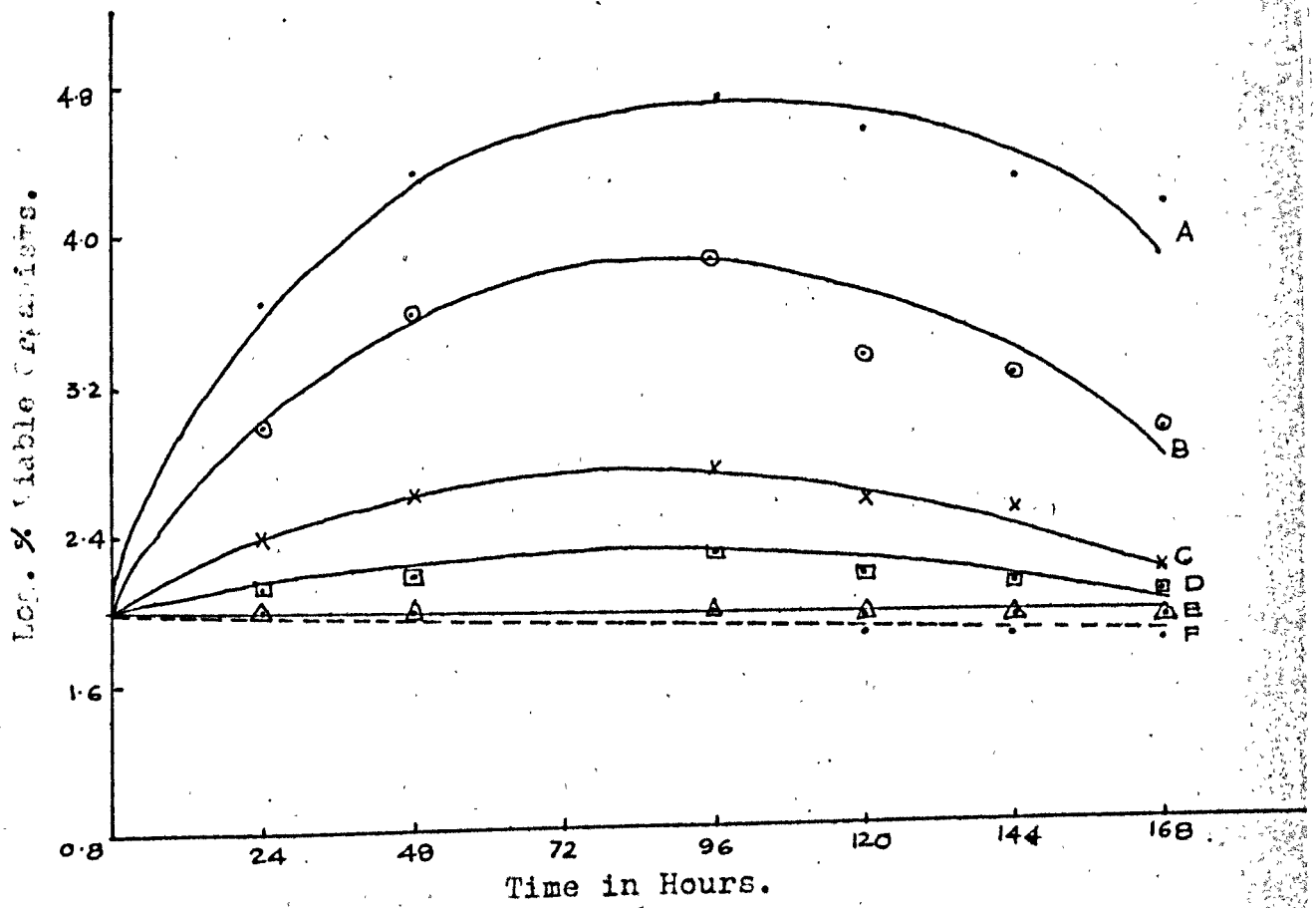


Fig. XIII Relationship between Amount of Eluate Obtained from Different Concentrations of Bacterium coli in Oil and the Growth Rate of the Organisms.

A:- Approximately $15x10^6$ organisms in oil.
B:- Approximately $12x10^6$ organisms in oil.
C:- Approximately $9x10^6$ organisms in oil.
D:- Approximately $6x10^6$ organisms in oil.
E:- Approximately $3x10^6$ organisms in oil.
F:- Control Ringer's solution.

approximately 15 x 10^6 viable organisms (i.e., total inoculum of about 750 x 10^6 organisms) there was in 96 hours a 500-fold increase of a standard inoculum introduced into the Ringer's solution. When the oil inoculum was 9 x 10^6 viable organisms (i.e., 450 x 10^6 total number of organisms) there was in 96 hours a ten-fold increase in the standard inoculum, and when the oil inoculum was only 3 x 10^6 (i.e., 150 x 10^6 total number of organisms) no multiplication was observed in the Ringer's solution. The rate of death of the organisms in the Ringer's solution was, however, much slower than was normally observed for Bacterium coli in Ringer's solution (Table 29). Thus, if the eluate from a small number of cells did not support multiplication in the Ringer's solution, it did alter the shape of normal time-survivor curve for Bacterium coli in Ringer's solution.

(K) THE DETERMINATION OF STABILITY OF THE NUTRITIVE ELUATE FROM BACTERIUM COLI.

The stability of the eluate from Bacterium coli was determined by heating Ringer's solution containing the eluate at 100°C for ½-hour, and subsequently inoculating with Bacterium coli. A control solution of eluate which was not heated was also inoculated. The two solutions were stored for 7 days and viable counts on both showed equal growth rates of the organisms (Tables 110-111) indicating that the eluate was not decomposed by boiling.

A volume of oil was inoculated with dry Bacterium coli and set aside for 48 hours, during which time the organisms died. A portion of the oil was then heated at 100°C and extracted with sterile Ringer's solution. An unheated portion of infected oil was also extracted with sterile Ringer's solution. Both portions of Ringer's solution were inoculated with Bacterium coli and stored for 7 days during which equal multiplication was observed in the two solutions (Tables 112-113). It would, thus, appear that the eluate obtained was stable in an anhydrous system at 100°C.

These observations confirmed the findings of Bean and Walters (1955) who obtained their eluate by boiling Bacterium coli with distilled water.

TABLE 110

MULTIPLICATION OF BACTERIUM COLI IN BOILED ELUATE.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of Ringer's solution
0 Hour	256	249	263	262	253	256.6	2.4092	2.8585	185,250
24 Hours	271	273	288	285	286	280.6	2.4481	3.8561	2,014,700
48 Hours	54	52	62	53	61	56.4	1.7513	5.3397	12,329,050
96 Hours	100	107	95	96	108	101.2	2.0051	5.9380	97,860,400
120 Hours	80	76	86	86	77	81.0	1.9085	5.9380	78,327,000
144 Hours	67	61	70	61	71	65.0	1.8129	5.6373	28,197,000
168 Hours	82	89	77	86	77	82.2	1.9149	5.3397	17,975,650

TABLE 111

MULTIPLICATION OF BACTERIUM COLI IN UN-BOILED ELUATE.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilu-tion Factor	Number of organisms per ml. of Ringer's solution
0 Hour	250	265	263	264	252	258.8	2.4130	2.8585	186,800
24 Hours	270	283	269	285	280	277.4	2.4429	3.8561	1,991,750
48 Hours	50	51	59	50	60	54.0	1.7324	5.3397	11,804,400
96 Hours	93	105	93	104	103	99.6	1.9983	5.9380	96,313,200
120 Hours	82	90	80	89	79	84.0	1.9243	5.9380	81,228,000
144 Hours	61	60	71	72	61	64.0	1.8062	5.6373	27,763,200
168 Hours	91	80	79	90	78	83.6	1.9159	5.3397	18,275,000

TABLE 112

MULTIPLICATION OF BACTERIUM COLI IN ELUATE OBTAINED FROM HEATED LIGHT LIQUID PARAFFIN.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml of Ringer's solution
0 Hour	262	269	256	253	255	259.0	2.4133	2.8585	186,950
24 Hours	280	290	275	275	289	281.8	2.4499	3.8561	2,023,300
48 Hours	52	59	59	51	60	56.2	1.7497	5.3397	12,285,550
96 Hours	106	110	100	97	107	104.0	2.0170	5.9380	100,568,000
120 Hours	80	76	87	87	85	83.0	1.9191	5.9380	80,261,000
144 Hours	76	69	76	64	64	69.8	1.8439	5.6373	30,279,250
168 Hours	77	89	78	82	88	82.8	1.9159	5.3397	18,100,100

TABLE 113

MULTIPLICATION OF BACTERIUM COLI IN ELUATE OBTAINED FROM UNHEATED LIGHT LIQUID PARAFFIN.

Duration of Storage	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Number of organisms per ml. of Ringer's solution
0 Hour	244	259	246	258	246	250.6	2.3991	2.8585	180,900
24 Hours	266	282	281	265	264	263.6	2.4210	3.8561	1,892,650
48 Hours	55	62	65	54	55	58.2	1.7649	5.3397	12,722,500
96 Hours	92	103	101	91	94	96.2	1.9832	5.9380	93,025,400
120 Hours	82	88	80	78	90	83.6	1.9222	5.9380	80,841,200
144 Hours	74	63	74	63	75	69.8	1.8439	5.6373	30,279,250
168 Hours	81	89	77	77	88	82.4	1.9159	5.3397	18,012,64

(L) THE NATURE OF ELUATE OBTAINED FROM BACTERIUM COLI.

Light Liquid Paraffin was infected with dry "uncoated" Bacterium coli and the organisms were allowed to die in 48 hours. The infected oil was shaken vigorously with sterile Ringer's solution and centrifuged at 8300 g., for 10 minutes. The oil was pipetted off and the supernatant Ringer's solution collected and recentrifuged at 8300 g., for 10 minutes. The ultra-violet absorption spectrum of the clear Ringer's solution containing eluate was examined using a Unicam S.P. 500 spectrophotometer. Ultra-violet absorption spectra of two control solutions, (a) sterile Ringer's solution and (b) Ringer's solution shaken with sterile light liquid paraffin, were also examined (Fig. XIV).

No absorption of ultra-violet was recorded over the range of 230-300 mμ in either of the two control solutions. In the Ringer's solution shaken with infected oil (containing eluate) there was a maximal ultra-violet absorption at a wave-length of 260 mμ. These results were similar to those obtained by Bean and Walters (1955). Thus, it appears that the eluate which supported growth of Bacterium coli in the present work was similar in nature to that studied by Bean and Walters (1955).

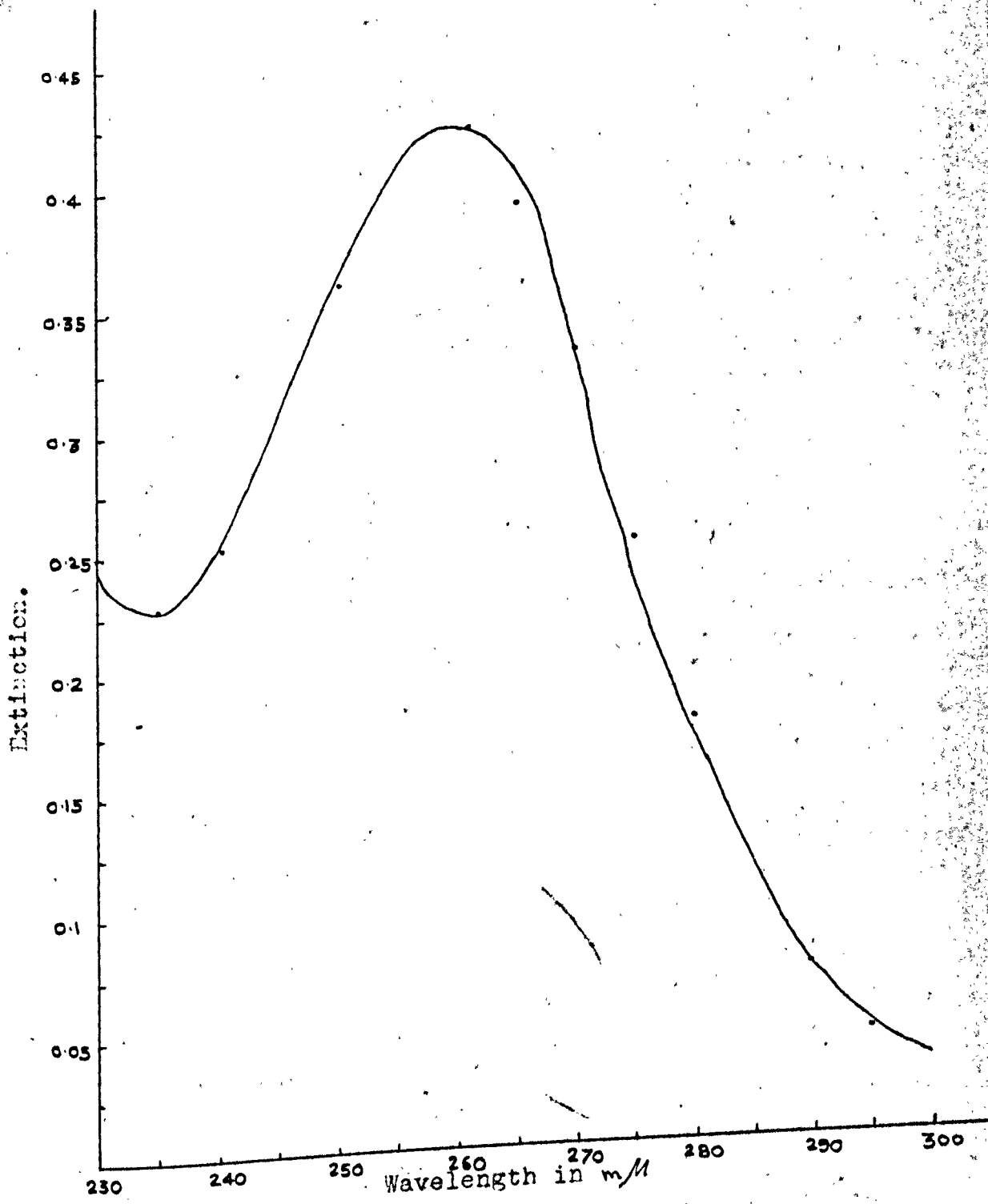


Fig. XIV Ultra-violet Absorption Spectra of Cell-free Eluate obtained from Infected Oil.

(M) THE MULTIPLICATION OF BACTERIUM COLI IN ARACHIS OIL-RINGER'S SOLUTION SYSTEM.

It has been conclusively shown above that the eluate released from the cells of Bacterium coli, after the organisms died in the oil, supported growth of the remaining viable organisms in an adjacent aqueous phase. A similar multiplication should occur when light liquid paraffin is replaced by any other fixed oil which is bactericidal to the organism.

Arachis oil was infected with dry "uncoated" Bacterium coli and viable counts performed on the suspension. Fifteen millilitres of this suspension were floated over sterile Ringer's solution in a series of separators. The Ringer's solution was collected from the separators after specified time intervals and viable counts performed (Table 114)

After the two phases had been in contact for 96 hours, there were approximately 18 times as many viable organisms in the Ringer's solution as were initially present in the oil. Most of the viable organisms introduced into the arachis oil died within 6 hours and all of them died within 24 hours (Table 46). A similar rate of multiplication during storage was observed in the Ringer's solution collected after 24 hours contact with infected oil and stored up to 7 days (Table 115). The observed multiplication in the stored Ringer's solution

TABLE 114

MULTIPLICATION OF BACTERIUM COLI IN INFECTED ARACHIS OIL-RINGER'S SOLUTION SYSTEM.

Initial number of viable organisms in oil :- 13,372,000.

Duration of contact between two phases	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Viable number of organisms in Ringer's solution
24 Hours	122	114	127	126	115	120.8	2.0820	5.0319	12,998,100
48 Hours	122	112	125	114	124	119.4	2.0770	6.1173	156,414,000
96 Hours	77	90	79	82	89	83.4	1.9212	6.5159	273,552,000
120 Hours	51	59	60	60	52	56.4	1.7513	6.5159	184,992,000
144 Hours	77	86	75	79	85	80.4	1.9053	6.1173	105,324,000
168 Hours	63	71	61	62	71	65.6	1.8169	6.1173	85,936,000

TABLE 115

MULTIPLICATION OF BACTERIUM COLI IN RINGER'S SOLUTION COLLECTED AFTER 24 HOURS CONTACT WITH ARACHIS OIL INFECTED WITH DRY "UNCOATED" BACTERIUM COLI.

Initial number of viable organisms in oil :- 13,372,000.

Duration of Storage after Contact	Counts of 5-replicate tubes					Mean Count	Log. Mean Count	Log. Dilution Factor	Viable number of organisms in Ringer's solution
0 Hour	122	114	127	126	115	120.8	2.0820	5.0319	12,998,100
24 Hours	116	123	112	123	121	119.0	2.0755	6.1173	155,890,000
72 Hours	80	79	91	90	79	83.8	1.9232	6.5159	274,864,000
96 Hours	49	58	59	48	51	53.0	1.7243	6.5159	173,840,000
120 Hours	74	72	82	82	75	76.0	1.8808	6.1173	99,560,000
144 Hours	61	68	59	68	61	63.4	1.8021	6.1173	83,054,000

indicated clearly that the organisms multiplied in the Ringer's solution after passing into it from the oil. The rate of multiplication was similar to that observed in the case of light liquid paraffin.

Thus, it can be concluded that the type of oil used has no relationship to the multiplication of Bacterium coli when the infected oily phase is brought into contact with an aqueous phase. The multiplication depends solely on the materials released by the organisms on their death.

SECTION IV.

SUMMARY OF DISCUSSION.

Since Ficker (1898) reported the bactericidal action of water containing metallic impurities, the deleterious effects of distilled waters from metallic stills have been reported by Wilson (1922), Hoder (1932), Davis (1940) and other workers. Such a deleterious effect of water from a metallic still was confirmed during the course of the present studies. The improved viability of Bacterium coli in Ringer's solution, observed during the present studies, confirmed the observations of Wilson(1922), Berry and Michaels (1947), Withell (1938) and others.

There is an extensive literature on the survival of organisms in aqueous systems, but comparatively little has been published on the survival of organisms in fixed oils. The majority of workers, with the notable exception of Bullock et al (1951-1953), who have undertaken the study of bacterial survival in oils, added aqueous suspensions of organisms to the oils. Their studies were in fact, concerned with the survival of organisms in aqueous droplets surrounded by an oily phase. In the work described in the present communication, freeze-dried organisms were used and thus the organisms were in direct contact with oil.

The protective effects of colloidal and other suspending agents on organisms during freeze-drying, and

during subsequent storage of the dry organisms, have been reported by Stamp (1947), Fry (1951) and Proom (1951). When Bacterium coli was freeze-dried from peptone solution containing no sodium chloride, about 25-30 per cent of the organisms survived the freeze-drying process, whereas only about 3 per cent survived when the same organism was freeze-dried from a suspension in distilled water. The addition of sodium chloride to the peptone solution was observed to have a lethal effect during the freeze-drying process. This lethal effect was attributed to the high osmotic pressure produced by concentration of the fluid during freeze-drying, which resulted in disruption of the cells.

The protective effect of nutritive materials on dry organisms was further illustrated when the organisms were treated with an organic solvent. A nil mortality was observed when Bacterium coli, freeze-dried from peptone solution, was treated for 20 minutes with petroleum ether. There was 88 per cent mortality when the same organism was freeze-dried from suspension in distilled water and similarly treated with petroleum ether. This indicated clearly that, in the case of "protected" organisms, the organic solvent probably never came into contact with the surface of the organisms. The latter were probably embedded in a nutritive coating which, being hydrophilic, prevented the penetration of a hydrophobic organic solvent.

Bullock and Keepe (1951) demonstrated that, of several organic solvents examined, petroleum ether was the only solvent which had no lethal effect on the spores of Bacillus subtilis in spray-dried peptone powder, even when the time of contact was extended to 90 minutes. The experiments recorded in the present work showed that spores of Bacillus subtilis suffered nil mortality when freeze-dried from suspension in distilled water, during 100 days subsequent storage or when dry "uncoated" spores were treated with petroleum ether for 20 minutes.

A nutritive coating on dry Bacterium coli was observed to protect the dry organisms from the bactericidal action of light liquid paraffin. The bactericidal activity of the oil was much more marked when the organisms were not protected by a nutritive coating. About 8 per cent of the viable "coated" organisms survived for 48 hours in light liquid paraffin, whereas there were nil survivors after 24 hours when dry "uncoated" Bacterium coli was suspended in either light liquid paraffin or arachis oil. The bactericidal action of light liquid paraffin was much more marked when oily suspension of the "uncoated" organisms was diluted. In fact, a linear relationship was observed between the number of the organisms in the oil and the mortality rate. Such a relationship between the concentration of the organisms and

the velocity of the bactericidal reaction has been reported previously for aqueous systems, but not for an oily system.

As might have been expected spores of Bacillus subtilis were more resistant to the oils than Bacterium coli. They did, however, die gradually, less than 10 per cent surviving 100 days storage in oils.

The observations, that oils are bactericidal to both vegetative cells and spores, are completely at variance with those of Bullock and Keepe (1951). These two apparently contradictory observations may be reconciled in the knowledge that, whereas the organisms used in the experiments described in the present communication were mostly free from adherent nutritive materials, those used by Bullock and Keepe (1951) were probably coated with dried peptone, and thereby protected from the bactericidal action of the hydrophobic oils. The present studies indicate clearly that fixed oils are unquestionably highly bactericidal to vegetative cells and mildly bactericidal to spores. This conclusion is supported by numerous sterility tests, performed by the British Pharmacoepoeia method, on samples of oils obtained from a dispensary. On one occasion only was a contaminant found and that was a sporing organism.

When light liquid paraffin was infected with dry

"coated" Bacterium coli and floated over sterile Ringer's solution, all the organisms sedimented through the oil and passed into the Ringer's solution within 30 minutes. It was concluded from these results that the organisms were not present as discrete free-living cells but as large "particles" which probably consisted largely of peptone. When an oily suspension of dry "uncoated" spores of Bacillus subtilis was floated over sterile Ringer's solution, the rate of sedimentation of the spores through the oil suggested that the organisms were present as large aggregates rather than as discrete cells. The aggregates were, however, much smaller than those of "coated" organisms. Viable counts on samples of dilutions of an oily suspension of spores confirmed that the suspension was heterogeneous. Sedimentation rates through the oil further suggested that some of the aggregates adhered to each other during sedimentation and formed larger aggregates which sedimented much more rapidly as the volume of the aggregates increased. It is probable that the presence of such aggregates of varied size in a suspension of dry "uncoated" Bacterium coli in oils is responsible for the observed variation in the viability of the organisms in the oils. (Page 138).

Attempts to improve the uniformity of such a heterogeneous suspension were made by removing the larger

aggregates by filtration. The suspension after filtration was much more uniform than before filtration. This was clearly revealed by the more uniform viable counts which were obtained on the samples of dilutions of the filtered suspension. The filtered suspension was naturally more dilute and contained only about 3 per cent of the number of organisms originally in the oil. The size of the aggregates in the filtered suspension was less than one-tenth of the size of those in the unfiltered suspension. None of the earlier workers seem to have realised that their suspensions probably consisted of large aggregates of varied sizes distributed in the oil. It appears that suspensions of free individual cells in oil have not yet been produced. The technique described in the present communication does, however, appear to produce reasonably uniform suspensions.

Only a comparatively small proportion of the organisms sedimenting through the oil penetrated into underlying Ringer's solution, while majority of them were adsorbed at the interface because they were "wetted" with the oil. Attempts were made to lower the interfacial tension and thus increase the number of organisms passing from the oil into the aqueous phase. A linear relationship was found to exist between the interfacial tension and the percentage of the organisms

initially in the oil passing into the aqueous phase. Minimal interfacial tension was obtained when the Ringer's solution contained 0.8 per cent Tween 80. Higher concentrations of Tween 80 were not employed because they produced no further reduction of the interfacial tension and, moreover, were bactericidal. Nevertheless, the present work indicates that if an oil infected with organisms is floated on an aqueous phase, it should be possible, by sufficiently reducing the interfacial tension, to permit all the organisms originally in the oily phase to pass into the aqueous phase.

The observation that some of the organisms sedimenting through the oil did penetrate into the lower aqueous phase suggested a new technique for estimating viable organisms in oils. An oily suspension of spores of Bacillus subtilis was centrifuged at high speed over an aqueous phase, and it was found from viable counts performed on aqueous phase that all the spores passed from the oil to the aqueous phase. The viable counts on the aqueous phase could thus be regarded as an estimate of the number of viable organisms in the oil. The counts thus obtained were usually somewhat higher than those given by the method of Bullock and Keepe, but the difference was not sufficiently great to be significant.

The application of the centrifuging method to an

oily suspension of dry "uncoated" Bacterium coli resulted in only about 40 per cent of the viable organisms in the oil passing into the aqueous phase in viable state. An investigation on the relation between time of centrifuging and viable counts on the Ringer's solution revealed a progressive death of the organisms with a progressive rise of temperature in the centrifuge. Subsequent experiments confirmed that while Bacterium coli in Ringer's solution suffered nil mortality within ½ hour at room temperature, the organisms died rapidly in Ringer's solution at 45°C.

The proposed centrifuging method for enumerating viable organisms in oils is shown to be perfectly satisfactory for spores. Furthermore, the experiments suggest that it would be equally satisfactory for vegetative organisms if it were possible to control the temperature of the centrifuge, as could be done by the use of a refrigerated centrifuge.

About 45 per cent of the viable Bacterium coli initially introduced into the oil sedimented under gravitational influence to the interface in 6 hours, but only about 10 per cent of the initial number of viable organisms in the oil penetrated into the Ringer's solution during the same period. During this period of 6 hours most of the organisms would have been killed in the oil. Nevertheless, after 48 hours contact

between the two phases, the number of viable organisms obtained in the aqueous phase was about 10-12 times the number of viable organisms originally introduced into the oil. The only interpretation that can be placed on this observation is that, whereas the organisms died in each of the two phases when separate, a multiplication occurred somewhere in the system when the two phases were brought together.

The multiplication was shown to occur in the aqueous phase. Multiplication of organisms can only occur in the presence of nutritive material, and the source of this material was shown to be the organisms themselves. When the organisms died in the oil, water-soluble materials were released from the cells. These materials went into solution in the aqueous phase, and thereby supplied nutrition to the remaining viable organisms which divided and multiplied. This multiplication of Bacterium coli in an oil-aqueous system was also observed when light liquid paraffin was replaced by arachis oil. Thus, the type of oil had no influence on the multiplication of the organisms, which depended solely on the eluate released by the organisms at death.

The rate of multiplication was observed to depend on the number of organisms originally in the oil and consequently on the amount of eluate obtained in the aqueous phase

when the infected oil was shaken with Ringer's solution. When the number of organisms in the oil was small, and consequently the amount of eluate in the aqueous phase was small, there was no multiplication of the organisms, but the survival of the organisms was prolonged in the aqueous phase. Thus, in lower concentrations the eluate seems to protect the organisms against the mildly bactericidal action of the aqueous fluid.

It was observed in the present studies that a relationship existed between the concentration of Bacterium coli in distilled water and the death-rate in that fluid. In higher concentration a smaller percentage mortality would result in a sufficient number of organisms being killed to produce a concentration of eluate high enough to protect the remaining viable organisms. The death-rate would thereby be reduced. In lower concentration, the amount of eluate released would be too small to protect the organisms. It seems probable that the reduction of mortality rate observed at the distal portions of time-survivor curves, which has been attributed to the increased resistance of the last survivors, is in fact, probably influenced by the release of cellular eluate which protects the last survivors.

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